IMMUNOMODULATION BY HEAT STRESS AND SOMATOTROPIN IN DAIRY COWS: RELEVANCE FOR THE EPIDEMIOLOGY OF MASTITIS

By

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To my parents and Bernard

To Dawn, Maghan and Kristin

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IMMUNOMODULATION BY HEAT STRESS AND SOMATOTROPIN IN DAIRY COWS: RELEVANCE FOR THE EPIDEMIOLOGY OF MASTITIS

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Effects of heat stress and recombinantly derived bovine somatotropin (bST) on immune function were investigated. Several effects of elevated in vitro incubation temperatures on isolated leukocytes could be measured, but similar effects generally could not be measured after in vivo heat stress. Incubation at 42°C, compared to 38.5°C, reduced function of polymorphonuclear leukocytes (PMNL) in assays of migration, oxidative metabolism and phagocytosis. Inhibiting effects of elevated temperatures in vitro were most noticeable in lymphocyte proliferation assays, in particular when elevated temperatures were applied during the cell activation phase. Heat stress in vivo increased numbers of leukocytes in peripheral blood leukocytes in one of two experiments, reduced sensitivity of lymphocytes to heat stress in vitro, but did not alter lymphocyte populations and subpopulations,

as evaluated by flow cytometry after fluorescent staining. In vitro chemotaxis of PMNL was reduced by in vivo heat stress. Oyster-glycogen stimulated migration of leukocytes into the mammary gland was inhibited, although, when no challenge was applied, somatic cell counts in milk increased.

No effects of in vitro or in vivo bST treatment on in vitro PMNL function were measured. At pharmacological doses (1000 ng/ml), bST in vitro had mitogenic effects on lymphocytes at 38.5 and 42°C, and in vitro and in vivo treatment with bST conferred some thermotolerance to lymphocytes after mitogen stimulation. Lymphocyte populations and subpopulations in peripheral blood were not altered by bST treatment, but total numbers of leukocytes, in particular of lymphocytes, were reduced.

Inflammation events in the mammary gland of lactating cows in summer and fall, treated or not treated with bST, were determined in a new approach for the evaluation of somatic cell count time series. Incidence of inflammation events was higher in summer than in fall. Duration of inflammation events was longer in bST treated cows, while severity of inflammation was not affected by season or bST.

Overall, heat stress reduced some functions of the immune system. Bovine somatotropin did not affect function of polymorphonuclear leukocytes but was mitogenic and conferred some thermotolerance to lymphocytes.

CHAPTER 1
REVIEW OF LITERATURE

The major function of the immune system is the protection against disease, particularly disease of infectious origin. Additionally, components of the immune system interact with physiological mechanisms controlling growth and reproduction. Traditionally, the immune system has been divided into humoral and cellular branches, but advances in biological sciences have made this separation obsolete. Indeed substances from the humoral branch of the immune system originate in large part from cellular components of the immune system, and interaction and complementation are essential for the proper functioning of the whole system.

Besides maintaining nonspecific immune mechanisms like phagocytosis, the body also has at its disposal an adaptive immune system, whose major features are memory, specificity, and recognition of nonself (Roitt, 1980). Social, meteorological, and other environmental factors can influence the immune system by enhancing or depressing its component parts. Numerous attempts have been made to understand and to manipulate the immune system to prevent or cure disease. Of particular relevance to this thesis are alterations in the

immune system by heat stress, which greatly depresses productivity of domestic food animals during warm months, and by recombinantly derived somatotropin, which will be used extensively to enhance growth and lactation of food animals. It is likely that these two factors influence the proper functioning of the immune system, with heat stress depressing and somatotropin possibly enhancing the function of the immune system. Indeed, important interactions may occur upon the combined exposure of cattle to heat stress and supplementation with recombinantly derived bovine somatotropin (bST). In this thesis the effects of these factors on the bovine immune system will be examined with special reference to parts of the mammary gland defense mechanisms.

Differentiation and Function of Immune Cells

The immune system includes 3 major types of cells, lymphocytes, monocytes, and polymorphonuclear leukocytes. These three cell populations can be divided into several types and subtypes by function, location, and expression of surface proteins.

Lymphocytes originate from a common hemopoietic stem cell in the bone marrow, and differentiate via a lymphoid progenitor cell into different cell types, B-cells, T-cells, and non-T, non-B cells or "null" cells. Further differentiation and maturation occurs in thymus (T lymphocytes) and in secondary lymphatic organs (lymph nodes and spleen). B and T lymphocytes, after stimulation with an

antigen, have the potential to develop memory cells, which can be reactivated by any subsequent challenge with the same antigen.

The major function of B-cells is the synthesis and secretion of immunoglobulins. Upon presentation of an antigen, generally on the surface of a macrophage, a signal is transmitted via membrane immunoglobulins to stimulate B-cell proliferation, differentiation into plasma cells, and secretion of immunoglobulins (reviewed by Cambier et al., 1987). B-cell activation requires stimulation by mediators secreted by monocytes (interleukin 1) and T_{helper} lymphocytes (interleukin 2, 4 and 5; reviewed by Anderson and Hill, 1988; Nonnecke and Harp, 1989). The major function of T lymphocytes is regulation of the immune response through synthesis and secretion of mediators. Three major sub-populations have been differentiated, the T_{helper}-, $T_{suppressor}$-, and $T_{cytotoxic}$-cells. T_{helper}- cells promote proliferation of B- and T-cells by secretion of lymphokines. They are stimulated by interleukin 1 and by antigen presenting cells, which are macrophages and small B lymphocytes, in context with class II major histocompatibility antigens (Lanzavecchia, 1987). $T_{suppressor}$ lymphocytes downregulate T and B lymphocyte responses in context with class I major histocompatibility complex (Dorf and Benacerraf, 1984; Asherson et al., 1986). The function of $T_{cytotoxic}$-cells is the defense against intra-cellular pathogens, i.e. viruses (Gommard et al., 1978), and are directed against

haptens or viruses presented in association with class I major histocompatibility antigens (Zinkernagel and Doherty, 1974; McMichael, 1978).

Lymphocytes can be differentiated by analysis of population-specific proteins expressed on the cell surface. B lymphocytes can be detected by the presence of surface membrane bound immunoglobulins, while T lymphocytes are generally differentiated by several cluster differentiation antigens expressed on the cell surface. Before the development of monoclonal antibodies to these antigens, T-cells were separated from B lymphocytes by their ability to form rosettes with sheep erythrocytes (Wilson et al., 1986). This sheep erythrocyte receptor has been characterized as cluster differentiation antigen CD3 and characterizes all T lymphocytes, as does CD2 (Davis et al., 1987). CD4 and CD8 glycoproteins are T-cell specific surface glycoproteins which are expressed on functionally distinct populations of T lymphocytes. CD4 is expressed on T_{helper}-cells and is not detected on $T_{suppressor}$- or $T_{cytotoxic}$-cells, which express CD8 (Reinherz and Schlossman, 1980; Engleman et al., 1981; Baldwin et al., 1986; Ellis et al., 1986).

Monocytes and polymorphonuclear leukocytes originate, as do lymphocytes, from a common hemopoietic stem cell and then differentiate via the myeloid cell lineage into effector cells. Monocytes, and their tissue counterparts (also called macrophages, dendritic cells, Kupfer cells, depending on their

location), take up antigens by phagocytosis, and once activated, secrete factors to induce migration of polymorphonuclear leukocytes (Craven 1983) or mediators to activate B lymphocytes and T lymphocytes (reviewed by Anderson and Hill, 1988; Nonnecke and Harp, 1989). The major function of macrophages is processing and presentation to T lymphocytes of antigen in association with major histocompatibility antigens (Steward, 1989).

The major function of polymorphonuclear leukocytes is phagocytosis of debris and pathogens. Recently, four polymorphonuclear leukocyte subpopulations have been described in the bovine, based on cell surface receptor expression (Paape et al., 1989). In general polymorphonuclear leukocytes attach to and ingest particles coated (i.e., opsonized) with immunoglobulins and complement factor C3. Polymorphonuclear leukocytes express surface receptors for the F_c-portion of the immunoglobulins and for C3b. To eliminate invading microorganisms, polymorphonuclear leukocytes are chemically stimulated (chemotaxis) to migrate towards the location where the contamination occurred. The arrival of the cells at the inflammatory site involves margination to the walls of the blood vessels and adherence to the endothelium, diapedesis (i.e. insertion of pseudopodia between endothelial cells and dissolution of the basement membrane), and directed migration along a chemotactic gradient (Hayashi et al., 1974; Lentnek et al., 1976). Generally two characteristics of migratory

function of polymorphonuclear leukocytes are evaluated in the laboratory, which are random migration and chemotaxis (Keller et al., 1977). Random migration is random in direction, and not oriented towards a stimulus, while chemotaxis is determined by substances in the environment which attract cells and stimulate them to move along a chemotactic gradient. Complement factors C3a and C5a are chemotactic agents (Fernandez et al., 1978). Craven (1983) found that bovine macrophages secrete a factor which attracts bovine polymorphonuclear leukocytes. Once polymorphonuclear leukocytes reach the microorganisms, phagocytosis, which involves attachment, engulfment, and killing of the microorganism takes place. Phagocytic activity can be evaluated by measuring oxidative metabolism of the cells. Indeed oxygen consumption increases during phagocytosis in a process called oxidative burst. Several oxygen metabolites are produced, including superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide (reviewed by Badwey and Karnovsky, 1980). These four compounds have antimicrobial properties, and are active within the cell, but are also excreted into the environment. An oxidative burst also occurs when macrophages are activated (Pabst and Johnston, 1980).

To summarize in a simplified schematic, invading pathogens generally first are phagocytosed by macrophages. Macrophages secrete a chemoattractant for polymorphonuclear leukocytes and mediators which lead to the activation of the

lymphoid system. Interleukin 1 activates T_{helper}-cells which then express interleukin 2 receptors, secrete interleukin 2, and proliferate. Interleukin 1 and 2 activate B-cells to mature, to proliferate, and to secrete immunoglobulins. Immunoglobulins and complement factors are required to opsonize microorganisms for phagocytosis by polymorphonuclear leukocytes.

Effects of Heat Stress

Heat Stress and Production

Mammals and birds maintain homeothermy through continuous and balanced exchange of heat. An animal is in homeothermy, when metabolic heat production is in equilibrium with heat loss to the environment, i.e., when net effects of heat gain equal net effects of heat loss. Homeothermic temperature ranges from 35.5°C in the shrew to 40°C in the hedgehog (Rodbard, 1950). Small birds have homeothermic temperatures up to 43.5°C. Normal body temperature in cattle is around 38.5°C. The maintenance of the body at such high temperatures allows homeotherms to maintain a more active metabolism than poikilotherms but also makes the homeotherms more sensitive to elevated high temperatures, since homeothermic temperatures are already close to temperatures at which enzymes begin to lose their activity due to the denaturing effects of elevated temperature. Not surprisingly, death can occur if body temperature exceeds homeothermic temperature by more than a few degrees.

Heat gain occurs through combustion of energy from feed and body stores, and from the environment. Animals release heat directly to their environment. Heat gain from and loss to the environment occurs in 5 modes of energy transfer (Curtis, 1983). Animals exchange heat through sensible heat transfer in processes of convection, conduction, and radiation. For all sensible heat loss, rate of heat loss is proportional to the difference between body temperature and environmental temperature. Animals lose heat by evaporation from the skin and the respiratory tract, and can gain heat by condensation. Heat stress to animals and resulting strain in tropical climates can occur through excessive radiation or through inhibition of loss of heat energy through conduction, convection, and evaporation.

At temperatures ranging within a thermoneutral zone, animals do not need to spend energy to maintain homeothermy. For dairy cows these temperatures range between 5 and 25°C (McDowell, 1972), and a rapid drop in milk yield occurs above 29°C (Rodriquez et al., 1985). Milk yields, rectal temperatures, and respiration rates are correlated to the temperature humidity index and to the black-globe humidity index. The latter takes into account incident solar radiation and is a good indicator of heat stress (Buffington et al., 1981). Cows maintained in a no shade environment with average black globe temperatures of 36.7°C produced about 10% less milk than cows maintained in a shade environment at average

black globe temperatures of 28.4°C (Roman-Ponce et al., 1977). Depression of milk production due to heat stress likely results in large part from depressed feed intake. Cows in no shade environment ate less during daytime (0800-1600h, -56%), more during nighttime (1600-0800h, +19%), and less total feed (-13%) than cows maintained in shade (Mallonée et al., 1985).

Heat Stress and Pathogen Survival

During summer months, the numbers of episodes of clinical mastitis (Roman-Ponce et al., 1977; Morse et al., 1988) and somatic cell counts in milk increase (Paape et al., 1973; Wegner et al., 1976; Bodoh et al., 1976; Bray et al., 1989). The increased incidence of mastitis in summer could be due to direct effects of high temperatures and humidities on the host or on growth of pathogens. High humidity favors the survival of bacteria and this is influenced by precipitation and moisture holding capacities of the environment (Wray, 1975). In buildings for dairy cattle, hemolytic staphylococci and Escherichia coli were the major organisms collected from the air (Fiser and Svitavsky, 1973, cited in Donaldson, 1978). Staphylococcus aureus survived best in relative humidities of 95-98%, and multiplication was highest at 30°C (McDade et al., 1963), while at 20°C, death rates of Staphylococcus aureus and β-hemolytic streptococci increased with increasing relative humidity (Lidwell and Lowbury, 1950). Thus, in hot and humid climates, there is potential for increased pathogenic challenge to the health of dairy cows. It also is apparent

that the resistance of the host may be depressed during heat stress such that pathogens can establish an infection more easily.

Immune Function During Hyperthermia

Increased body temperature may affect the immune system by altering peripheral blood cell numbers and cell differentiation. Contradictory results have been reported. Heat exposure of Holstein calves for 7 d did not affect total peripheral blood cell count but decreased percentage of neutrophilic polymorphonuclear leukocytes and increased percentage of lymphocytes (Kelley et al., 1982b). Wegner et al. (1976) and Berning et al. (1987) found that total numbers of blood leukocytes increased when cows were subjected to elevated temperatures in hyperthermic chambers, while their lymphocyte to neutrophil ratio decreased. Regnier and Kelley (1981) reported decreased total leukocyte numbers in blood of heat-stressed chickens.

Heat stress and resulting hyperthermia may affect function of cells. Heat exposure of Holstein bull calves reduced several indicators of cell-mediated immune responses such as expression of delayed-type hypersensitivity reactions after sensitization with heat-killed Mycobacterium tuberculosis, contact sensitivity reactions to 1-fluoro-2,4-dinitrobenzene, and phytohemagglutinin induced skin test reactions (Kelley et al., 1982a). Contact sensitivity and delayed-type hypersensitivity reactions express previously

acquired immune responses, which involve migration of lymphocytes, predominantly T lymphocytes, followed by a nonspecific inflammatory reaction with invasion of polymorphonuclear leukocytes. Similar effects could be measured in chickens (Regnier and Kelley, 1981). Kelley and coworkers (1982b) did not detect alteration of proliferation of lymphocytes in vitro from heat-stressed calves. Serum obtained from heat-stressed calves, when added to a lymphocyte culture from a nonstressed donor calf, enhanced concanavalin A and phytohemagglutinin-stimulated proliferation of cells compared to lymphocytes incubated in the presence of serum from nonheat-stressed calves. This could be due to the presence of a factor stimulating T-cell proliferation in the serum of mildly heat-stressed calves. Immunoglobulin G_1 (IgG_1) secretion is reduced by 25% in heat-stressed calves compared to control calves in thermoneutral environment (Kelley et al., 1982b). Heat-stressed calves also have higher mortality rates (Stott et al., 1976).

Results are different for lymphocytes obtained from human volunteers subjected to artificial hyperthermia. Proliferation of lymphocytes after stimulation with phytohemagglutinin and with staphylococcal enterotoxin B was depressed in cells from heat-stressed subjects, and proliferation was further depressed if cells were incubated in the presence of post-hyperthermia autologous plasma as compared to pre-hyperthermia autologous plasma (Downing and Taylor, 1987). In the same

subjects, natural killer cell activity was increased by the elevation of body temperature, as was in vitro interleukin 2 production after phytohemagglutinin stimulation. The same group found an increase in the proportion of $T_{suppressor/cytotoxic}$ lymphocytes, and a decrease in the percentage of T_{helper}-cells (Downing et al., 1988), due to heat stress. In mice, production of interleukin 1 was reduced for 4 h after 1 h of whole body hyperthermia at 41.5°C (Neville and Sauder, 1988), and was also reduced when culturing human peripheral blood adherent monocytes at 41°C (Schmidt and Abdulla, 1988). Interleukin 1 induces B lymphocyte proliferation and acts to activate T lymphocytes to secrete interleukin 2, and to express interleukin 2 receptors for clonal expansion (Smith and Rusceti, 1982). Thus, the reduction in interleukin 1 production could be a central event in changed cell-mediated immune responses in heat-stressed animals.

The direct effect of elevated temperature on lymphoid cells varies with degree of hyperthermia. At temperatures characteristic of mild hyperthermia, elevated temperatures in vitro can exert positive effects on the function of certain components of the immune system. Incubation of human lymphocytes at 39°C (as compared to homeothermic 37°C) led to an increased proliferation after stimulation with phytohemagglutinin, concanavalin A, and pokeweed mitogen (Närvänen et al., 1986). As compared to cells incubated at 37°C, incubation of human mononuclear cells at 38.5°C enhanced

incorporation of ^{3}H-thymidine after stimulation with phytohemagglutinin, while no change in proliferation occurred when cells were incubated at 40°C (Roberts and Steigbigel, 1977). Enhanced proliferation could also be shown in murine thymocytes, stimulated with concanavalin A in the presence of interleukin 1 and 2 (Duff and Durum, 1983). Pre-incubation of mouse T_{helper}-cells at 39.5°C enhanced their activity as compared to T_{helper}-cells pre-incubated at 37°C, while $T_{suppressor}$-cell activity was not altered (Jampel et al., 1983). In other studies, synthesis of leukocyte migration inhibition factor by lymphocytes was not changed (Närvänen et al., 1986), or was enhanced (Roberts and Sandberg, 1979). Production of IgM, IgG, and IgA by lymphocytes stimulated with pokeweed mitogen was reduced in 39°C incubation temperatures (Närvänen et al., 1986), as was B lymphocyte proliferation induced by lipopolysaccharide stimulation (Duff and Durum, 1983; Ciavarra et al., 1987). Proliferation of mouse T lymphocytes, induced by alloantigens, or by Sendai virus, a murine pathogen, was diminished at febrile temperatures, while the opposite was true for concanavalin A stimulated cells (Ciavarra et al., 1987). Proliferation of the Lyt-$1^{+}23^{-}$ lymphocyte subset, which represents T_{helper}-cells, was also reduced.

The effects of factors on function of polymorphonuclear leukocytes are more difficult to evaluate because of the short functional life span of those cells in culture (Washburn et al., 1982). Roberts and Steigbigel (1977) detected a slight,

yet significant enhancement of killing of Escherichia coli, Salmonella typhimurium, and Listeria monocytogenes by human polymorphonuclear leukocytes at 40°C vs 37°C. Killing of Staphylococcus aureus was not altered. Bactericidal capacity of mononuclear phagocytes did not change.

Thus it appears that different leukocyte populations and subpopulations react differently to a mild elevation of temperatures in vitro. Differences could be due to different abilities of cells to secrete heat shock proteins. THP-1 cells, a myelomonocytic cell line, increased synthesis of 70- and 90-kDa heat shock proteins already at 39°C, while human peripheral blood adherent monocytes required a temperature of 41 to 42°C to induce synthesis of heat shock proteins (Schmidt and Abdulla, 1988). Mizzen and Welch (1988) showed that exposure of human, rat embryo, and hamster cells to a severe but relatively short heat stress resulted in some resistance to damage caused by a subsequent severe heat stress. In heat tolerant cells, synthesis of 70-kDA heat shock proteins was increased. It is not clear whether heat shock proteins themselves confer thermotolerance. Heat shock proteins have been detected after activation of lymphocytes by phytohemagglutinin in non-heat-stressed cells (Haire et al., 1988). Kaczmarek et al. (1987) related the synthesis of heat shock proteins to different growth stages of peripheral blood mononuclear cells.

No enhancement of immune function has been reported when cells were exposed to severe heat stress, and in fact, most studies note a large decrease in immune function. Exposure of human mononuclear cells to 42.7°C for 2 hours subsequently led to a more than 50% reduced protein synthesis, with and without stimulation by phytohemagglutinin (Roberts, 1986). Human natural killer cell activity was nearly abolished by exposure to 42°C for one hour (Kalland and Dahlquist, 1983). B- and T-cells exposed to 45°C for one hour before stimulation with phytohemagglutinin incorporated less than 10% of ^{3}H-thymidine incorporated by unheated controls, while protein synthesis was reduced by more than 75%. Exposure of lymphocytes to elevated temperatures also resulted in the appearance of $T_{suppressor}$-cells in mixed lymphocyte cultures (Loertscher et al., 1987).

There are several possible explanations why heat-stressed animals would have a depressed immune function. One possibility is that elevated temperature in hyperthermic animals directly disrupts function of immune cells. Also, the depression in feed intake associated with heat stress (Mohammed and Johnson, 1985) could conceivably result in deficiencies of vitamin A and E, which could contribute to depression of immunological capacity (Boyne and Arthur, 1979; Arthur et al., 1981; review on vitamin A in Chew, 1987; Tjoelker et al., 1988a,b). Heat stress led to a rise in circulating concentrations of glucocorticoids (Christison and Johnson, 1972; Wise et al., 1988), which could depress

function of polymorphonuclear leukocytes and lymphocytes. Indeed, treatment in vitro with various levels of hydrocortisone (Ojo-Amaize et al., 1988) and in vivo with adrenocorticotropin (Roth et al., 1982), hydrocortisone, or dexamethasone (Muscoplat et al., 1975; Phillips et al., 1987) caused a depression of bovine lymphocyte blastogenesis after stimulation with Staphylococcus aureus capsular extract, phytohemagglutinin, or concanavalin A. Function of polymorphonuclear leukocytes could also be reduced. Although Phillips et al. (1987) could not detect effects of in vitro or in vivo dexamethasone treatment on chemiluminescence by polymorphonuclear leukocytes, Roth and Kaeberle (1981) found, apart from enhanced chemotaxis, that ingestion of Staphylococcus aureus, oxidative metabolism, and antibody-dependent, cell-mediated cytotoxicity by bovine polymorphonuclear leukocytes were reduced after in vivo treatment with dexamethasone. McGillen et al. (1980) detected reduced adherence of human polymorphonuclear leukocytes to nylon wool after in vitro treatment with hydrocortisone. Christison and Johnson (1972) found that concentrations of cortisol in plasma returned to normal when mild heat stress became chronic (after 7 to 10 weeks), while Wise et al. (1988) measured continuously elevated concentrations of cortisol in heat-stressed cows during a whole estrous cycle.

In summary, elevated temperatures can alter immune responses. Large increases in temperature depress the immune

system, while effects of mild hyperthermia depend on the component tested. It is important to note that during heat-stress periods, lactating cows can display very severe hyperthermia. Wegner et al. (1976) reported an increase of average rectal temperature in 4 cows to 43.1 ± 0.5°C (rectal temperature at homeothermy: 38.5°C), and elevated temperatures by more than 3°C are frequently reported in heat-stress trials. Thus it is of interest to evaluate how components of the immune system are affected by this level of heat stress, using in vitro and in vivo models. It is also of interest to evaluate whether adaptation and tolerance of the immune system to high environmental temperature and relative humidity will occur. Adaptation could be due to an alteration of leukocyte cell populations (Downing et al., 1988), or through protective mechanisms like synthesis of heat shock proteins (Mizzen and Welch, 1988).

Effects of Somatotropin

Secretion, Treatment, and Production

Growth hormone is a single chain polypeptide, containing about 191 amino acid residues and 2 disulfide bridges. It is produced predominantly by acidophilic cells in the anterior pituitary gland, but production of somatotropin has also been detected in rat lymphocytes (Weigent et al., 1988). Secretion of growth hormone is pulsatile and is regulated by stimulatory and inhibitory hypothalamic factors (Girard, 1984). Human growth hormone releasing factor, somatocrinin, isolated from

a pancreatic tumor, stimulated growth hormone secretion in rat and man (Guillemin et al., 1982), and in cattle (Moseley et al., 1984; Lapierrre et al., 1988). Somatostatin, with 14 amino acid residues, has been isolated by Brazeau et al. (1973) and has been shown to inhibit release of growth hormone in sheep and rats (Davis, 1975; Abe et al., 1983). Yousef et al. (1969) calculated a growth hormone secretion rate in nonlactating cows of 19.1 mg per day per animal to maintain average concentrations of growth hormone in plasma of 15 ng/ml. Hart et al. (1980) found lower secretion rates and basal plasma concentrations in dry cows (0.6 μg/min/100 kg, 3.0 ng/ml), and determined that secretion rates and concentrations of somatotropin in high yielding cows are higher at d 30 of lactation (1.6 μg/min/100 kg, 5.5 ng/ml), than at d 90 (1.1 μg/min/100 kg, 3.5 ng/ml) or d 150 (0.6 μg/min/100 kg, 1.7 ng/ml). In low yielding cows, secretion rates and plasma growth hormone concentrations were less (Hart et al., 1980). Trenkle et al. (1972) calculated a secretion rate of 47 ng per kg per day to maintain average levels of 18.0 ng/ml plasma in 3 month old bull calves. Plasma somatotropin levels decreased with increasing age to 7.1 ng/ml for bulls and 4.7 ng/ml for heifers, while Schams et al. (1980) measured higher concentrations in prepubertal heifers than in prepubertal bulls. Values of 1 to 6 ng somatotropin per ml plasma are generally reported in lactating cows (Peters

et al., 1981; Hart et al., 1985; Igono et al., 1988; Lough et al., 1989).

Injections of native pituitary and recombinantly derived bovine somatotropin to lactating dairy cows increased milk yields in short- and long-term experiments by 8 - 40 % (Peel et al., 1981; Richard et al., 1985; Bauman et al., 1985). In long-term trials, injections were accompanied by increased intake of feed energy (Chalupa et al., 1986) and resulted in increased gross efficiency of milk production (Soderholm et al., 1988; reviewed in Chalupa and Galligan, 1989). Injected dosages of bST in efficacy trials ranged up to 100 mg/d, and lowest effective doses during full lactation trials have been 6.25 mg/d or greater (Eppard et al., 1985; Elvinger et al., 1988). Injection of 30 to 40 mg of bST increases levels of growth hormone in plasma of cows to 10-30 ng/ml (Hart et al., 1985; Lough et al., 1989). Effects of bovine somatotropin are reviewed by Chilliard (1988) and Bachman et al. (1990).

Responses to bST injections may vary because of different stages of lactation and producing abilities of cows, or due to different management practices and environments. Smaller increases in milk and constituent yields were recorded when treatments were administered during environmental heat stress (Mohammed and Johnson, 1985; Zoa-Mboe et al., 1986; Elvinger et al., 1988). The reason for smaller increases may be associated with reduced intake of feed resulting from heat stress.

Somatotropin and Health

Concentrations of somatotropin in plasma which are attained by injections of 6-40 mg bST/d, or 500 mg bST every two weeks are not expected to be toxic in treated cows. Indeed no adverse effects on cow health could be detected in chronic toxicology studies, where cows received 1800 and 3000 mg bST bi-weekly for up to 2 lactation cycles (Eppard et al., 1988; Cole et al., 1988). Also in acute toxicology studies (Vicini et al., 1988), where cows received 15,000 mg twice in a 2 week period, no negative effects of somatotropin were observed, even though concentrations of somatotropin in plasma were greater than 250 ng/ml. Higher serum levels of somatotropin are expressed in transgenic mice (up to several microgram per milliliter; Palmiter et al., 1983) and result in severe hepatic and renal lesions (Quaife et al., 1989). A contributing factor to the dysfunctions observed in transgenic animals is that those hormones are secreted in organs where secretion does not occur under physiological conditions. Also the timing of excessive secretion may be of importance, since transgenic animals display elevated growth hormone levels during fetal development, which may be the cause for the non-allometric growth of liver and spleen (Quaife et al., 1989). Thus effects observed in transgenic animals expressing high serum levels of growth hormone cannot be projected to occur in adult cows treated to maintain elevated serum levels of much lower amplitude.

No pathological changes have been detected in short- and long-term bST trials in dairy cows. Bovine somatotropin does not alter rectal temperatures, respiration rates, or heart rates when cows were maintained within thermoneutral zone and under sound management (Eppard et al., 1987). In most studies, the number of leukocytes in peripheral blood is slightly elevated, but within physiological ranges, and is due to either an increase in number of polymorphonuclear leukocytes or lymphocytes (Eppard et al., 1987; McGuffey et al., 1990). Numbers of somatic cells in milk were not affected by treatment, and there was no evidence for elevated incidence of mastitis due to treatment with bST (Elvinger et al., 1988).

Somatotropin and the Immune System

The effect of bST on milk yields is indirect, i.e., milk yield increases are mediated through increased levels of insulin-like growth factors I and II (Dehoff et al., 1988), and these molecules have no known effects on the immune system (Berczi, 1986). Nonetheless, there is evidence for direct effects of somatotropin on cells of the immune system in the bovine and in other species. Receptors for human growth hormone have been detected on human lymphocytes (Lesniak et al., 1974; Kiess and Butenandt, 1985, 1987; Asakawa et al., 1986; Smal et al., 1987), and bovine and murine thymocytes display receptors for bovine growth hormone (Arrenbrecht, 1974). Recently, growth hormone production by cells of lymphoid origin has been reported: rat and human lymphocytes

produced immunoreactive growth hormone which was similar to pituitary somatotropin in terms of bioactivity, antigenicity, and molecular weight (Weigent et al., 1988). This growth hormone enhanced incorporation of [^{3}H]-thymidine up to 10-fold in Nb2 node lymphoma cell cultures (Weigent et al., 1988).

Whitfield and coworkers (1971) demonstrated that addition of growth hormone to rat thymic lymphocytes promoted their progression into the S-phase of the proliferation cycle. Humoral and cell mediated immune reactions were impaired by hypophysectomy (Nagy and Berczi, 1978) and human and bovine growth hormone restored the immunological properties of hypophysectomized rats (Nagy et al., 1983). Subcutaneous injections in hypophysectomized rats with somatotropin increased the antibody response to sheep red blood cells to levels measured in non-hypophysectomized rats and also reconstituted the inflammatory response to skin sensitization with dinitrochlorobenzene. In immunocompromised aged rats, injections of bovine growth hormone (750 ng two times daily for 5 weeks) increased proliferation of lymphocytes in vitro after stimulation with concanavalin A and phytohemagglutinin (Davila et al., 1987).

Some results contest the positive effects of somatotropin on components of the immune system. No effects of physiological concentrations of human growth hormone, added in vitro, on thymidine incorporation after 6 or 24 h incubation by lymphocytes from 11 out of 17 children with acute

lymphoblastic leukemia could be detected (Blatt et al., 1987). This lack of effect may have been due to the short incubation time (6 h) in the presence of growth hormone in this experiment. Kiess et al. (1988) found that natural killer (NK) cell activity in growth-hormone-deficient patients was lowered, but the authors were not able to restore NK cell activity by treatment with growth hormone releasing factor. Also, no changes in proportions of T lymphocytes, T_{helper}- and $T_{suppressor}$-cells, B lymphocytes, and natural killer cells could be detected. Proportions of all lymphocyte types and subtypes were within normal ranges. Rapaport and coworkers (1986) reported that treatment of 7 growth hormone deficient children with human growth hormone for 12 months did not affect serum IgG, IgA and IgM, , or % T lymphocytes, T_{helper}- or $T_{suppressor}$-cells. The percentage of B lymphocytes decreased below subnormal levels, $T_{helper}/T_{suppressor}$-cell ratios decreased, and proliferation of lymphocytes was reduced when cells were stimulated with phytohemagglutinin in vitro. In another report, the same authors (Rapaport et al., 1987) found that the mitogen-induced proliferative response of lymphocytes from growth-hormone-deficient children could be enhanced by treatment in vivo with growth hormone.

There are no reports as to whether cells of myeloid origin have growth hormone receptors and effects of growth hormone on those cells have not been extensively investigated. Treatment with human growth hormone of patients with pituitary

dwarfism increased the reductase activity of granulocytes isolated from peripheral blood at resting conditions and after stimulation of phagocytosis by starch, and a slight increase in oxidative metabolism was observed after adding growth hormone to granulocytes (Rovensky et al., 1982, reported in Berczi, 1986). Chemiluminescence, a measure of oxidative burst, and chemotactic migration under agarose of polymorphonuclear leukocytes were not altered after growth hormone treatment of growth-hormone-deficient children (Rapaport et al. 1986). On the other hand, the incubation of mononuclear phagocytes derived from porcine blood with native and recombinant porcine somatotropin led to an 18-fold increase in production of superoxide anion after stimulation with zymosan (Edwards et al., 1988). Production of superoxide anion by polymorphonuclear leukocytes from dairy cows was increased 5 to 8 days after the start of treatment with bST (Heyneman et al., 1989).

Thus it appears that somatotropin can affect immune function, but that effects of somatotropin treatment depend on species, component of the immune system, and immune status of the treated animal. Lactating cows are expected to have physiologically normal growth hormone levels, and their immune system is expected to be sufficient. Since gross efficiency of lactation is increased by bST treatment (Chalupa and Galligan, 1989), intake of nutrients influencing the immune system may be altered, and management procedures should insure

that use of bST does not result in deficient levels of Se, vitamin A or E, or other feed ingredients in the rations, which could have a negative effect on the immune system (Boyne and Arthur, 1979; Arthur et al., 1981; Tjoelker et al., 1988a,b). Reports on effects of somatotropin on immune status of healthy individuals or on effects of in vitro somatotropin treatment on components of the cellular immune system are scarce. It is of interest to understand how chronic bST treatment affects the immune system of dairy cows and their resistance to infectious diseases.

Heat Stress and Somatotropin

Somatotropin secretion patterns are altered during heat stress. In swine (Marple et al., 1972) and rats (Parkhie and Johnson, 1969), heat stress increased circulating concentrations of growth hormone. In contrast, somatotropin concentrations in serum did not increase in calves and lactating cows exposed to high temperatures (29-35°C) and relative humidities of 55-60% (Schams et al., 1980; Mohammed and Johnson, 1985), although concentrations of somatotropin in milk, but not in plasma, increased from 6.8 to 9.4 ng/ml (Mohammed and Johnson, 1985). Igono et al. (1988) found that growth hormone concentrations in milk decreased with increasing temperature humidity index for cows yielding more than 10 kg milk per day. There was no clear relationship in cows producing less than 10 kg per day. Cows exposed to 35°C secreted less somatotropin (9 mg per day per animal) than cows

exposed to 18°C (16 mg per day per animal; Mitra et al., 1972), and although turnover rate of somatotropin was reduced at high temperature, i.e., half-life of somatotropin in circulation increased, growth hormone concentrations in plasma from heat-stressed cows were lower than in cows in thermoneutral environment (Mitra et al., 1972).

While bST improved lactation yields in hot and humid environments (Mohammed and Johnson, 1985; Elvinger et al., 1988), decreases of milk yield caused by high temperatures increased with bST treatment doses (Elvinger et al., 1988). High producing cows were less responsive to bST when treated in hot conditions (West et al., 1989). Injection of 200 or 300 mg of somatotropin to cows increased heat production by 30 to 40% at an ambient temperature of 22°C, and by 50 to 60% for cows maintained at 38°C (Yousef and Johnson, 1966). Somatotropin also increased O_2 consumption by 22% at 18°C to 29% at 38°C. The latency period between injection of bST and increased heat production was 10 hours at 18°C, but less than 4 hours at 38°C (Yousef and Johnson, 1966). The period of increased metabolic rate was longer at elevated temperature, perhaps because heat stress decreased turnover of somatotropin (Mitra et al., 1972).

Yousef and Johnson (1966) measured an increase in rectal temperatures from 38.8 to 40.1°C after injection of growth hormone, even at an ambient temperature of 18°C, a temperature within the thermal comfort-zone. This finding was not

duplicated in short- and long-term trials with bST (Eppard et al., 1985; Johnson et al., 1988; Manalu et al., 1988). Zoa-Mboe et al. (1989) and West et al. (1989), however, measured increased body temperature in bST-treated cows during summer. This could be due to the difficulty of cows to dissipate metabolic heat in heat-stress environment. The large increase in body temperature measured by Yousef and Johnson (1966) could be due to the high doses utilized in that experiment.

Effects on the Immune System

Interactive effects of heat stress and somatotropin on the immune system have not been documented. Nonetheless, there is some evidence that certain types of stress-induced changes in immune function can be alleviated by somatotropin. Since stress is associated with release of adrenocorticotropic hormone (ACTH) and adrenal glucocorticoids, many effects of stress can be simulated by injections of these hormones. When cortisol was injected to hypophysectomized rats, leukocyte counts in peripheral blood were decreased by up to 50% (Chatterton et al., 1973). When rats were simultaneously treated with growth hormone, no leukopenia occurred and number of leukocytes even increased by up to 30%, primarily due to higher numbers of polymorphonuclear leukocytes. Injections of growth hormone alone in intact or hypophysectomized rats did not elicit an increase in leukocyte counts (Chatterton et al., 1973). In another study (Hayashida and Li, 1957), antibody response to a Pasteurella pestis antigen was depressed in

hypophysectomized rats by ACTH injections and could be restored by treatment with somatotropin. As compared to non-treated controls, antibody titers of somatotropin-treated non-ACTH-treated animals were higher, while rats treated with ACTH and somatotropin had antibody titers nearly as high as non-treated controls (Hayashida and Li, 1957).

Although treatment with somatotropin frequently improves function of components of the immune system in patients with immunodeficiencies, effects of heat stress on the immune system of cows treated with bST are difficult to predict, since several metabolic changes take place. In some ways, somatotropin exerts effects on the immune system antagonistic to those of heat stress. For example, depression of delayed-type hypersensitivity and contact sensitivity skin reactions were reduced by heat stress in calves (Kelley et al., 1982a), and were restored by treatment with somatotropin in rats (Nagy et al., 1983). Also, in vitro proliferation of lymphocytes, depressed by in vivo heat stress (Downing and Taylor, 1987), could be enhanced by somatotropin treatment (Davila et al., 1987; Whitfield et al., 1971). Heat stress and bST-treatment both tend to improve function of polymorphonuclear leukocytes (Roberts and Steigbigel, 1977; Heyneman et al., 1989), at least under mild heat-stress conditions (39 vs 37°C). Thus it remains to be determined how heat stress and bST interactively affect components of the immune system.

Immune System of the Mammary Gland

The teat canal is considered to be the first line of defense of the udder for protection from invading pathogens (reviewed by Craven and Williams, 1985). A keratin plug, which contains bactericidal free-fatty-acids, constitutes a mechanical and chemical barrier (Chandler et al., 1969; McDonald, 1971a,b; Morse et al., 1971). Leukocytes are present in sub-epithelial and epithelial teat end tissues (Nickerson and Pankey, 1983). The predominant leukocyte type are lymphocytes, which could respond to antigens in the proximal teat canal and in the area of the Fürstenberg rosette by producing antibodies and lymphokines. Once pathogens penetrate into the teat cistern, a second line of defense (Paape, 1979) by components of the phagocytic immune system is activated. As early as 1906, Rullmann and Trommsdorff reported that milk from mammary quarters infected with Streptococcus agalactiae had increased volumes of leukocyte pellets obtained by centrifugation. Relative volumes increased from less than 0.1% in uninfected quarters to 8.5% in infected quarters having an average of 6.5×10^6 colony forming units of Streptococcus agalactiae. Milk from healthy noninfected udders contains less than 100,000 cells. The predominant type of cells in milk from uninfected quarters are macrophages (Lee et al., 1980), which represent about 60% of cells. Polymorphonuclear leukocytes represent 12%, and 28% are lymphocytes. Milk from infected quarters can contain

several million cells per milliliter, of which more than 90% are polymorphonuclear leukocytes (Oldham et al., 1989).

Cells of Myeloid Origin in the Mammary Gland

Initiation of the cellular immune response depends on the proper functioning of macrophages and polymorphonuclear leukocytes. The major protective function in the mammary gland of polymorphonuclear leukocytes is phagocytosis of invading pathogens. When cows were made deficient in polymorphonuclear leukocytes, infection of the bovine udder with Staphylococcus aureus resulted in gangrenous mastitis and death (Jain et al., 1968; Schalm et al., 1976). When stimulated by a chemoattractant, polymorphonuclear leukocytes migrate from capillaries and venules, accumulate in the subepithelial connective tissues, and then cross the epithelium into the teat cistern (Nickerson and Pankey, 1984) or into the alveoli (Harmon and Heald, 1982). It does not appear as if bacteria or their products act as chemoattractants for bovine polymorphonuclear leukocytes (Gray et al., 1982; Carrol et al., 1982; Bruecker and Schwartz, 1982; Craven, 1983). Components of the complement cascade, C3a and C5a, may act as chemoattractants, but Mueller et al. (1983) estimated that complement levels in milk were insufficient for induction of chemotaxis. A likely source for a chemoattractant are macrophages, which secreted a chemoattracting factor when activated with pre-opsonized Staphylococcus aureus (Craven, 1983, 1986). In assays of

migration under agarose, this chemoattractant induced chemotaxis and chemokinesis of polymorphonuclear leukocytes. Migration of polymorphonuclear leukocytes can be initiated by infusion of sterile irritants like oyster glycogen (Paape et al., 1977) and lipopolysaccharides from several bacteria (Guidry et al., 1983). This property can be used to study treatment effects on migration of leukocytes to the mammary gland and also facilitates the collection of sufficient numbers of leukocytes for in vitro studies of function of milk somatic cells (Paape et al., 1977).

Although influx of cells during infection is massive, function of polymorphonuclear leukocytes is reduced in milk. Mammary macrophages and polymorphonuclear leukocytes phagocytosed less bacteria in milk because of ingestion of casein and fat (Duhamel et al., 1987; Dulin et al., 1988). Oxidative burst activity was reduced in polymorphonuclear leukocytes obtained from milk, as compared to cells obtained from blood (Weber et al., 1983; Dulin et al., 1988), and lactose exerted negative effects on response in chemiluminescence assays (Weber et al., 1983). Another reason for this depression could be the low concentrations of glucose as energy source in milk (Newbould, 1973).

On the other hand, antibody binding capacity of isotypes IgG_1, IgG_2 and IgA was higher in polymorphonuclear leukocytes recovered from the mammary gland than from blood (Berning et al., 1989). Binding of IgM was much lower. Polymorphonuclear

leukocytes phagocytose pathogens opsonized with IgG_1, IgG_2, IgM, or IgA (Brandon et al., 1981). These authors measured 0.6 mg IgG_1, 0.06 mg IgG_2, 0.09 mg IgM, and 0.13 mg IgA per ml bovine milk whey, although some IgA and IgM, but no IgG_1 and IgG_2 may be bound to the milk fat globule membrane (Honkanen-Buzalski and Sandholm, 1981). Phagocytosis by bovine neutrophils was promoted by IgG_2, but not by IgG_1 (McGuire et al., 1979), and Watson (1976) determined that IgG_2 was cytophylic for neutrophils (Fidalgo and Najjar, 1967). Immunoglobulin G_2 bound preferentially to polymorphonuclear leukocytes. Approximately 25% of polymorphonuclear leukocytes from ewes had complete IgG_2 (fractions F_c and F_{ab}), but not IgG_1, IgA, or IgM on their surfaces (Watson, 1976).

The high incidence rate of infections during early involution (Natzke, 1981; Oliver and Mitchell, 1983) may be related to reduced phagocytic ability of polymorphonuclear leukocytes and macrophages. The lowest phagocytic activity was measured for phagocytes from early dry period secretion, as compared to later dry period secretion, colostrum, and normal milk (Targowski and Niemialtowski, 1986; Fox et al., 1988). Although Holmberg and Concha (1985) reported reduced migratory capacity of leukocytes during the early dry period, they found that at the same time a higher percentage of cells phagocytosed latex particles. In mastitic milk, percentage of cells phagocytosing and number of bacteria phagocytosed were much lower than in normal milk (Targowski and Niemialtowski,

1986), and were further reduced by addition of antibiotics commonly used to treat mastitic quarters (Nickerson et al., 1985, 1986; Lintner and Eberhart, 1987).

Attempts have been made to determine if cell function itself is inhibited, or if secretions from dry or mastitic quarters contain factors which inhibit phagocytosis. Conflicting results have been obtained. Miller et al. (1985) measured highest phagocytosis in presence of secretion from early dry period, while Targowski and Niemialtowski (1986) reported depressed percentage of cells phagocytosing and number of Staphylococcus aureus phagocytosed in presence of early dry period secretion.

Nutritional factors may alter the migratory response of polymorphonuclear leukocytes, although it is not known if effects are exerted on macrophages or on polymorphonuclear leukocytes. Selenium and vitamin E depletion tend to result in a less massive migration of polymorphonuclear leukocytes to the udder after intra-mammary infusion of E.coli lipopolysaccharide (Hogan et al., 1989). Additionally, intracellular killing, but not phagocytosis, was reduced after Se and vitamin E deficiency. Bulk tank somatic cell counts decreased with increasing herd average of Se concentrations in plasma of cows (Weiss et al., 1990). Also, rates of clinical mastitis decreased when levels of Se and Vitamin E increased in the diet of cows in well managed herds.

Lymphocytes in the Mammary Gland

Function of lymphocytes in the defense of the mammary gland has not been elucidated and only recently did research efforts focus on their contribution to the protection of the udder from pathogens. The majority of cells recovered from dry udder secretions were lymphocytes, out of which 45-80% were T-cells, and 3-20% B-cells (Concha et al., 1978; Duhamel et al., 1987; Hurley et al., 1990). Prior to calving, percentage of B lymphocytes increased until it approximated the percentage of T lymphocytes. $T_{helper}/T_{suppressor}$-cell ratio was slightly lower for mammary gland lymphocytes (2.2:1) than for peripheral blood lymphocytes (2.7:1) and decreased towards parturition (1.6:1; Hurley et al., 1990). In mastitic cows, Yang et al. (1988) found an increase in percent B lymphocytes and a decrease in percent T lymphocytes in the supra-mammary lymph node, while the opposite occurred in the prescapular lymph node and in peripheral blood. Proliferation of milk lymphocytes after stimulation by mitogens was reduced compared to peripheral blood lymphocytes (Smith and Schultz, 1977; Nonnecke and Harp, 1985). Supernatants from milk lymphocyte cultures (Harp & Nonnecke, 1986) had inhibiting effects on proliferation of peripheral blood lymphocytes, especially when milk lymphocytes were isolated from quarters with chronic staphylococcal infections. Mammary gland mononuclear cells from dry gland secretions did not proliferate in response to phytohemagglutinin, concanavalin A or pokeweed mitogen (Schore

et al., 1981; Collins and Oldham, 1986), although interleukin-2 production by mammary gland lymphocytes after stimulation with concanavalin A nearly equaled production by peripheral blood lymphocytes (Collins and Oldham, 1986). Thus it appears that lymphocytes in dry gland secretions are unable to respond to the interleukin 2 stimulus in a manner sufficient for initiation of DNA synthesis.

Modulation of Immune Response

Attempts at enhancing the immune response in the mammary gland have been numerous. Traditionally efforts focused on modulation of the specific immune system by vaccination, while more recently, progress in recombinant gene technology has made available products like interleukin 2 and granulocyte-colony stimulating factor, which are being tested currently as immunoenhancing agents in the mammary gland.

The drawback to using vaccination to control mammary gland infections is that mastitis is a multifactorial disease, with many different pathogen species and strains. Vaccination attempts are generally directed against a particular pathogen. This renders the approach rather inefficient, unless a particular genus, species and strain is endemic in a herd, and economic losses due to mastitis can be traced to that pathogen. Nevertheless, research towards the development of vaccines is intense, and provides insight into mechanisms of the humoral immune system.

Vaccination attempts have been frequently directed towards control of infections by Staphylococcus aureus, a pathogen which is frequently resistant to antibiotic treatment. Vaccines to Escherichia coli also are being tested. Mastitis caused by Streptococcus agalactiae mastitis can be controlled efficiently by hygiene management procedures (Natzke, 1981; Elvinger, 1983), and thus does not generate the same interest for vaccination approaches.

Brock et al. (1975) were not able to elicit an immune response in cows by simultaneous intramuscular and intramammary vaccination with formalin-killed Staphylococcus aureus in Freund's complete adjuvant. Vaccination did not result in higher levels of anti-Staphylococcus aureus IgM, IgG1, IgG2, or IgA in serum, colostrum, or milk. Different approaches for antigen preparation have been taken, and different routes of immunization have been tested. Injections of a Staphylococcus aureus bacterin and staphylococcal hemolysins into the region of the external inguinal lymph node resulted in elevations of specific IgG in milk (Opdebeeck and Norcross, 1982). Subcutaneous injections of attenuated Staphylococcus aureus also elicited an immune response in heifers, increasing specific levels of IgG_1 and IgG_2 in serum (Watson, 1984). In case of inflammation caused by infusion of Escherichia coli endotoxin (Guidry et al., 1983), IgG_1 and IgG_2 both increased, but IgG_2 increased faster and by a larger relative amount than IgG_1. Since Watson (1975) determined

that IgG_2 was cytophilic and had opsonizing properties, it appears that stimulation of pathogen-specific IgG_2 secretion and transfer to the mammary gland, or eventually secretion and release of specific IgG_2 in the mammary gland (Newby and Bourne, 1977) should be enhanced to provide protection from invading pathogens.

Although immunized heifers became infected after challenge with Staphylococcus aureus, milk production did not decrease, as it did for non-vaccinated heifers (Watson, 1984). Pankey et al. (1985) immunized cows intramuscularly with a commercial Staphylococcus aureus bacterin, by injection in the region of the supra-mammary lymph node. Subsequently, teats were immersed regularly in pathogen suspensions after removal of the milking machine. No differences in incidence of Staphylococcus aureus infections were detected between control and immunized cows over a 3 year period, but rates of spontaneous recovery were higher in immunized cows.

As mentioned earlier, products obtained from recombinant gene technology have become available and may be useful for enhancing immune function in the mammary gland. Interleukin 2 and granulocyte-colony stimulating factor have been tested for their effects on the bovine immune system, with particular reference to the mammary gland immune system. Nickerson et al. (1989) surgically implanted mini-osmotic pumps releasing interleukin 2 into the teat cistern of cows at drying off. Half of the implanted quarters were previously infected with

Staphylococcus aureus, while the other half was free of infection. Interleukin 2 treatment resulted in the highest prevalence of lymphocytes, IgG_1 and IgG_2 positive cells, but lowest prevalence of IgA and IgM positive cells in previously infected quarters.

Recombinantly derived granulocyte-colony stimulating factor (G-CSF) enhanced in vitro functional activity of peripheral blood neutrophilic polymorphonuclear leukocytes collected from mastitic cows. Phagocytosis and luminol-enhanced chemiluminescence were increased in the presence of 5 ng/ml G-CSF, with an improvement of greater magnitude in neutrophils from mastitic cows (Reddy et al., 1989). In vivo effects of G-CSF on the immune system were evaluated in periparturient (Kehrli et al., 1990) and lactating cows (Nickerson et al., 1989). Peripheral blood leukocyte numbers were increased up to 5-fold, mainly due to increases in neutrophilic polymorphonuclear leukocytes, but numbers of mononuclear cells rose also. The relative percent of CD5 positive cells, which are mainly T lymphocytes, increased, but the ratio of $T_{helper}/T_{suppressor}$ lymphocytes did not change due to treatment (Kehrli et al., 1990). Somatic cell numbers in milk increased about two-fold in treated cows (Nickerson et al., 1989). Shedding of bacteria in Staphylococcus aureus infected quarters was not affected (Kehrli et al., 1990), but cows treated with G-CSF were more resistant to new experimental infections than non-treated cows (Nickerson et al., 1989).

Effects of Somatotropin and Heat Stress on the Immune System in the Mammary Gland

Should bovine somatotropin prove to be immunostimulating in cows, beneficial effects on the mammary gland immune system could be expected. Since most studies on effects of somatotropin have reflected the situation in immunodeficient animals, it is possible that positive effects of somatotropin on immune function will be observed in animals and cell cultures rendered immunodeficient by heat stress. There have been few reports available on function of the immune system of the mammary gland under adverse environmental conditions, and these have only considered changes in somatic cell counts. Although elevated somatic cell counts and increased incidence of clinical mastitis were measured in summer (Paape et al., 1973; Wegner et al., 1976; Bodoh et al., 1976; Bray et al., 1988; Morse et al., 1988), and although injections of adrenocorticotropic hormone led to increases in somatic cell counts (Wegner et al., 1971; Berning et al., 1987), no elevation of somatic cell counts in milk from healthy quarters could be measured when cows were exposed to heat stress in environmental chambers (Paape et al., 1973; Wegner et al., 1976; Berning et al., 1987).

Inhibition of the immune system in the mammary gland will not manifest itself necessarily by an elevation of somatic cell counts. It is likely that the opposite occurs. If the release of chemoattractants by macrophages is reduced, and/or if the migratory potential by polymorphonuclear leukocytes is

inhibited, then a slower and less massive infiltration by leukocytes will occur. This will be reflected in a lower somatic cell count at time of challenge, although a moderate elevation of somatic cell counts may persist for a longer period of time. In addition to decreased resistance, an increased pathogenic challenge seems to be necessary to produce an increase in SCC, respectively to increase incidence rate of clinical mastitis under heat stress conditions.

Somatic Cell Counts in the Diagnosis of Inflammation of the Mammary Gland

Variability of somatic cell counts reported from individual cows over time results from 4 major sources: intra-assay variation of the somatic cell counting procedure, time of sampling and amount of milk in the udder, age and stage of lactation, and inflammation events in the mammary gland, most generally due to contamination by pathogens and infection. This latter source of variation is responsible for a change in somatic cell counts of much greater magnitude than the other sources (Brolund, 1985).

Different procedures are used for somatic cell counting. Savage (1907) described a method to stain cells on slides and count them under a microscope. This method, modified by Prescott and Breed (1910), is used today as a reference procedure for electronic counting methods. Electronic particle counting (Coulter Counter^{R}), and fluoro-opto-electronic procedures (Fossomatic^{R}) are the most commonly used

methods (Booth, 1985). Storage time and temperature, and treatment of milk samples can influence electronic counts (Sweetsur and Phillips, 1976; Grear and Pearson, 1976; Dohoo et al., 1981a). Counts by electronic procedures are generally higher than the direct microscopic counts (Miller et al., 1986). This could be due to the fact that the Coulter Counter can count cell fragments, protein aggregates, and non-milk particles as cells (Hoare et al., 1982; Brooker, 1978), and Fossomatic considers large nuclear fragments as cells (Heald et al., 1977). Nevertheless, both electronic procedures yield repeatable results which are highly correlated to results from direct microscopic cell counts (Heeschen, 1975; Schmidt-Madsen, 1975; Szijarto and Barnum, 1984).

Time of sampling and amount of milk in the udder also influence somatic cell counts (Convey et al., 1971; Duitschaever and Ashton, 1972; Fernando and Spahr, 1983; Brolund, 1985). Slight differences in counts at morning and evening milking are due to unequal milking intervals. Somatic cell counts are highest in foremilk 3 h after milking, decrease to their lowest level about 9 h past milking, and then rise up slightly to the next milking. This is the case for infected and noninfected quarters, although amplitude of changes is higher in infected quarters (Fernando and Spahr, 1983). Therefore, as the interval between milkings decreases, milk yield also decreases, and this results in increased somatic cell counts.

Effects of lactation number and stage of lactation on somatic cell counts are controversial. Residual variance for the two effects amounted to 9.7% and 0.8% in all quarters, and 13.2% and 0.9% in quarters that were consistently bacteriologically negative (Brolund, 1985). Blackburn (1966) and Brooks et al. (1982) found that cell counts in milk from noninfected quarters increased with increasing lactation number, while Natzke et al. (1972b), Eberhart et al. (1979), and Bodoh et al. (1981) reported only slight increases. Sheldrake et al. (1983a) did not measure an increase due to lactation number in noninfected quarters, but found that cell counts increased with lactation number for quarters infected with Staphylococcus aureus, Staphylococcus epidermidis, and Corynebacterium bovis. Prevalence of infection increased with age (Bakken, 1981; Brooks et al., 1982). Therefore somatic cell counts could increase with increasing age because of higher prevalence of previously infected quarters or cows, or of infected quarters with negative bacteriological results (Brolund, 1985). Also, lactation stage can affect somatic cell counts (Ali and Shook, 1980). In several studies, somatic cell counts at the start of lactation were high, decreased within the first weeks, but then increased again towards the end of lactation (Cullen, 1968; Honkanen-Buzalski et al., 1981; Kennedy et al., 1982). This trend, however, could not be confirmed by Natzke et al. (1972b), Eberhart et al. (1979) and Brooks et al. (1982), while Emanuelson and

Persson (1984) found that trends due to lactation stage detected for unadjusted somatic cell counts could not be detected anymore when somatic cell counts were adjusted for milk yield.

The major determinant for somatic cell counts in milk is the presence or absence of mammary gland pathogens. The higher the somatic cell count, the larger the probability that a quarter is infected with a major pathogen (Eberhart et al., 1979). Somatic cell counts from uninfected quarters averaged 123,000 cells/ml (Mattila, 1985), while in infected quarters they were generally higher. Sheldrake et al. (1983a) reported somatic cell counts between 84 and 832×10^3 cells/ml for quarters infected with minor pathogens, which included Corynebacterium bovis and coagulase-negative staphylococci, and between 216 and $9,120 \times 10^3$ cells/ml milk for Staphylococcus aureus infected quarters. Ward and Schultz (1972) reported means of 770 to 2050×10^3 cells/ml milk in quarters infected with Streptococcus agalactiae and Streptococcus uberis. Infection and inflammation resulting in an increase in somatic cell count may affect one or more quarters (Natzke et al., 1972b). If composite milk samples were obtained from an udder inflamed in one quarter only, the dilution of the milk from that quarter by milk from the three healthy quarters would mask the somatic cell count increase in that quarter. This dilution effect is likely to be enhanced by the decreased milk yield of the inflamed quarter, and the concurrent compensating

increase in milk yield in the noninflamed quarters (Woolford, 1985). Nevertheless, with each new quarter infected in an udder, somatic cell counts in a composite milk sample increase (Bodoh et al., 1981), and are likely to double (Natzke et al., 1972b).

Duration of infection is variable and cell counts of established infections are generally higher than cell counts in milk from quarters with transient infections (Brolund, 1985). Responses to attempts at experimental infection of the mammary gland (Postle et al., 1978; Hill et al., 1978; Doane et al., 1987), ranged from no infection up to acute and life-threatening, as well as chronic infections. This illustrates that the processes caused by the invasion of pathogens are highly variable. The failure to develop an infection could be due to the inability of the pathogens to attach and to multiply in the new environment in case of reduced virulence (Frost, 1975, 1977; Doane et al., 1987), or to resistance of the cows to the pathogen. If invading pathogens are rapidly removed by polymorphonuclear leukocytes, then chronic rises of somatic cell counts may not occur. In case of chronic infections, number of pathogens and number of cells stay in a dynamic equilibrium, with periodic steep increases of somatic cell numbers and clinical manifestations, and periods with relatively low levels of somatic cells counts. The duration of a chronic infection is variable. Forbes and Herbert (1968) report that 11 of 32 Staphylococcus aureus infections

disappeared after 3 to 22 weeks, and 5 of 13 Staphylococcus epidermidis infections after 4 to 25 weeks. Also Brolund (1985) observed that the duration of infection with a particular pathogen is of limited duration. In 17617 quarters sampled twice, only 54% of quarters infected with Staphylococcus aureus detected in a first sampling were still positive at a subsequent sampling one month later. Recovery of other pathogens was lower, and 55% of quarters infected with Streptococci non-agalactiae were negative at the next sampling. In 13465 full-lactation records with recorded infections, out of 2030 spontaneously recovered cases, 20.1% lasted less than 6 weeks, and 27.7% persisted for 6 to 15 weeks (Natzke et al., 1972a, 1975).

Given the variable behavior of pathogens, and the variable response of the host, it is difficult to determine udder health status by somatic cell counts. Several variable thresholds for determining mastitis in cows have been proposed. Dohoo et al. (1981b), and Sheldrake et al. (1983b) suggested age-related or pathogen-related thresholds for classifying milk as mastitic. The problem with this approach is that somatic cell counts do not necessarily return to pre-infection levels, and therefore thresholds have to be kept high to avoid false-positive classifications. McDermott et al. (1982) have shown that prevalence of infection in a given population affects the sensitivity and specificity of diagnosis with a fixed threshold. When prevalence increases,

sensitivity of tests increases and specificity decreases. Mattila (1985) proposed the use of inter-quarter ratios to compare inflamed quarters with noninflamed quarters in the same cow. This method requires many samples and poses a problem in cows with continuous alterations due to previous infections and inflammations (Linzell and Peaker, 1974), or in cows with more than one quarter inflamed.

To be able to conclusively determine if somatic cell counts in milk are affected by heat stress and/or by bovine somatotropin, it is advantageous to develop a system to evaluate somatic cell counts with regard to variable responses to challenge, which also takes into account variation due to assay, diurnal variation, and long term trends. Frequent sampling during short periods of time may allow a better evaluation of challenges to the cow's udder and allow the assessment of the resistance status of the cow.

Conclusion

Heat stress and resulting hyperthermia generally depress the immune system. Somatotropin also affects the immune system, and immune function can be partially restored in growth hormone deficient and concurrently immune deficient patients by treatment with somatotropin. In tropical and sub-tropical climates cattle are exposed to heat stress, and bovine somatotropin is likely to exert a beneficial effect on the immune system. The objective of experiments for this dissertation was to evaluate whether heat stress and bST

affect components of the immune system with particular reference to the mammary gland immune system. Additionally, an epidemiological approach is taken to evaluate how season and supplemental bST affect frequency and characteristics of inflammation events in the mammary gland.

CHAPTER 2
MODULATION OF FUNCTION OF BOVINE POLYMORPHONUCLEAR LEUKOCYTES AND LYMPHOCYTES BY ELEVATED TEMPERATURES IN VITRO AND IN VIVO

Introduction

Heat stress increases the susceptibility of food animals to infectious disease (Webster, 1981). For example, the number of episodes of clinical mastitis and levels of somatic cell count (SCC) increased during summer (Paape et al., 1973; Wegner et al., 1976; Bodoh et al., 1976; Bray et al., 1988; Morse et al., 1988). It is likely that increases in disease incidence during hot and humid months occur in large part because the environment promotes proliferation and survival of pathogens (Lidwell & Lowbury, 1950; McDade & Hall, 1963; Wray, 1975). Host resistance to pathogens may also be reduced during heat stress. With increasing temperature and humidity, lactating cows loose the ability to dissipate heat derived from their metabolic activity and the environment, and body temperatures increase. As a result of this hyperthermia, a variety of physiological and cellular changes occur that could affect immunological resistance. Whole body hyperthermia has been reported to reduce interleukin 1 synthesis in mice (Neville & Sauder, 1988), to increase $T_{suppressor}$-cells and decrease percentage of T_{helper}-cells in healthy humans (Downing

et al., 1988), and to reduce the expression of delayed-type hypersensitivity reactions, contact sensitivity, phytohemagglutinin (PHA) induced skin test reactions and IgG_1 secretion in calves (Kelley et al., 1982a,b).

Elevated incubation temperature has been reported to affect several aspects of lymphoid cell function. For example, culture at 42.7°C for two hours reduced subsequent protein synthesis of resting and phytohemagglutinin-stimulated human mononuclear cells (Roberts, 1986). As compared to culture at 37°C, there was a reduction in secretion of IgM, IgG, and IgA by human lymphocytes, and proliferation of mouse B lymphocytes was reduced when cells were incubated at 39°C (Ciavarra et al., 1987; Duff & Durum, 1983; Jampel et al., 1983; Närvänen et al., 1986). An increase in incubation temperature reduced biosynthesis of p35, an interleukin 1B precursor protein, in human peripheral blood adherent monocytes, while heat shock protein (hsp70 and hsp90) synthesis was increased (Schmidt & Abdulla, 1988). At very high temperature (45°C), synthesis of a factor stimulating interleukin 2 secretion and expression of interleukin 2 receptors were reduced (Loertscher et al., 1987).

A low degree of hyperthermia could also enhance leukocyte function. Incubation at 39°C (vs 37°C) increased lymphocyte proliferation after stimulation with PHA, concanavalin A (conA), and pokeweed mitogen (PWM) of human lymphocytes (Närvänen et al, 1986) and of certain subsets of mouse T

lymphocytes (Ciavarra et al., 1987; Duff & Durum, 1983; Jampel et al., 1983). Kelley and coworkers (1982b) did not detect alterations in proliferation of lymphocytes of heat-stressed calves, but serum obtained from those calves, when added to lymphocyte cultures from a nonstressed calf, increased proliferation after stimulation with ConA and PHA to a greater degree than serum from nonstressed calves. Roberts and Steigbigel (1977) detected a slight, yet significant, enhancement of killing of Escherichia coli (E.coli), Salmonella typhimurium, and Listeria monocytogenes by human PMNL at 40°C vs 37°C, although killing of Staphylococcus aureus was not improved.

The objective of this study was to determine effects of elevated temperature in vitro on polymorphonuclear leukocytes and lymphocytes in cattle, and to evaluate whether similar changes were apparent after whole body hyperthermia.

Materials and Methods

Reagents

Dulbecco's phosphate buffered saline solution (DPBS), Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, cytochrome c from horse heart (Type III and VI), zymosan A, Histopaque 1077, phytohemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A (ConA), L-glutamine and trypan blue were purchased from Sigma Chemical Company (St. Louis, MO). RPMI-1640 was supplemented (modified RPMI-1640) with 1% (v/v) 200 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin

(GIBCO, Grand Island, NY), and 10% (v/v) bovine calf serum (HyClone, Logan, UT). [Methyl-^{3}H] thymidine (specific activity 5.0 Ci/mmol) was purchased from Amersham International (Arlington Heights, IL). Agarose was purchased from GIBCO (Grand Island, NY), Brain Heart Infusion Broth (BHI) from BBL Microbiology Systems (Becton Dickinson and Co., Cockeysville, MD) and a modified Wright's stain (Leukostat) from Fisher Diagnostics (Orangeburg, NY). Sodium dodecyl sulfate (SDS) was obtained from Hoefer Scientific Instruments (San Francisco, CA). A bovine serum pool was obtained from 5 Holstein cows. Blood agar was prepared with Bacto Tryptose Blood Agar Base from DIFCO Laboratories (Detroit, MI) and red blood cells obtained from Holstein cows.

Animals and Experimental Design

In vitro heat stress experiments were performed on isolated peripheral blood leukocytes from lactating Holstein cows. The effects of in vivo heat stress on leukocytes were evaluated in an experiment which utilized eight Holstein cows (2nd parity, 1st third of lactation), randomly assigned to two groups of four cows each. Each group was submitted to two environmental treatments in three periods of four days in a double reversal design. The treatments were thermoregulated environment (TR), in which cows were provided with shade and evaporative cooling facilities (sprinklers and fans turned on periodically) or heat-stress environment (HS), in which cows did not have access to shade or other cooling facilities. The

2 groups were subjected to the treatment sequences TR-HS-TR (Group 1) and HS-TR-HS (Group 2). The experiment began on September 8, and the maximal daily environmental dry bulb temperatures ranged from 31.1 to 36.1°C. Rectal temperature, respiration rate, and heart rate were monitored daily at 2 p.m. Blood, for the evaluation and isolation of leukocytes, and milk samples, for the evaluation of somatic cell counts, were collected on the last day of each period at 3 p.m. The isolated peripheral blood leukocytes were submitted to in vitro temperature treatments for measurement of function. Single milk samples for bacteriological evaluation were collected before, during and after the environmental treatment periods.

Intra-Cisternal Temperature Measurements

Intra-cisternal temperatures were measured with an intra-mammary temperature probe in one quarter of 4 lactating Holstein cows on one day at 8 a.m. Subsequently 2 cows were provided with shade and 2 cows were submitted to a no-shade environment. Intra-cisternal temperatures were again measured at 1 p.m. The temperature probe was constructed with a thermocouple wire inserted in reinforced polyethylene tubing of 1.5 mm diameter (Fisher Diagnostics, Orlando, FL), and connected to a handheld digital thermocouple thermometer (Model 450ATT, OMEGA Engineering Inc., Stamford, CT). Insertion depth could be measured with etchings on the surface of the probe, 1 cm apart from 1 to 10 cm.

Analysis of Milk Samples

Foremilk samples for bacteriological evaluation were obtained aseptically. Ten μl of milk per quarter were spread on 1/4 blood agar plate and examined for bacterial growth after incubation for 24 and 48 h at 38°C. For the microscopic evaluation of somatic cell numbers, 10 μl of milk were expanded on a surface area of 1 cm^2 of a microscopic slide and stained. Twenty microscopic fields were counted using a 40x lens of a light microscope, and somatic cell counts were calculated per ml milk (International Dairy Federation, 1981).

Blood Collection

Blood samples for the evaluation and isolation of leukocytes were obtained by veni-puncture, using evacuated heparinized blood collection tubes.

Leukocyte Count and Differentiation

Blood (20μl) was diluted with 380 μl 3% [v/v] formic acid to lyse red blood cells (RBC). Leukocytes in the resulting suspension were counted in a hemocytometer. For leukocyte differentiation, blood was spread on a microscopic slide and stained with modified Wright's Stain. One hundred cells were differentiated under the oil immersion lens of a light microscope to determine relative numbers of neutrophilic and eosinophilic polymorphonuclear leukocytes, and mononuclear cells (lymphocytes and monocytes).

Isolation of Leukocytes

Blood (10 ml) was diluted 1:1 in DPBS and the diluted blood was layered on 10 ml Histopaque 1077 and centrifuged for 30 min at 400 g. The packed red blood cell layer in the bottom, which also contained polymorphonuclear leukocytes, was collected and polymorphonuclear leukocytes were separated from red blood cells by hypotonic lysis: 10 ml dH_2O were added to 5-10 ml packed RBC and mixed. After 30 seconds, 10 ml 2x DPBS were added to restore isotonicity. The cell suspension was centrifuged at 400 g for 10 min, the supernatant discarded and remaining RBC were lysed by repeating the procedure. The final PMNL pellet was resuspended at 10^7 live cells/ml (viability determined by trypan blue exclusion) in DPBS.

For the isolation of lymphocytes, cells in the interface between Histopaque 1077 and plasma were collected, suspended in 5 ml DMEM, centrifuged for 10 min at 400 g, after which the cell pellet was resuspended in 4 ml DMEM and layered on 4 ml histopaque. After centrifugation for 30 min at 400 g, the interface was collected, washed 2x in 10 ml DMEM with centrifugation at 400 g for 10 min, and the cell pellet was resuspended in modified RPMI-1640 at 10^6 live cells/ml (viability determined by trypan blue exclusion).

Polymorphonuclear Leukocyte Functional Assays

Cytochrome c reduction was evaluated in a final volume of 1 ml DPBS containing 20 % serum and 80 mM cytochrome c (Rajkovic and Williams, 1985) by stimulating 3 x 10^6 PMNL with

4 mg opsonized zymosan. Zymosan was opsonized by preincubation of 20 mg per ml serum for 30 min at 38.5°C on a tube rotator. For in vitro experiments, all reagents and cells were normalized to assay temperature before mixing, while in the in vivo experiment, cells and reagents were mixed on ice. The reaction took place in test tubes incubated in a water bath at various temperatures. The reaction was stopped 5, 10 or 30 min after addition of opsonized zymosan by placing the test tubes on ice. Tubes were then centrifuged at 4°C for 10 min at 2600 g and absorbance of the supernatant was measured at 550 nm. The change in absorbance was calculated by subtracting values for control incubations maintained on ice during the incubation procedure.

The assays of phagocytosis and killing of E.coli by polymorphonuclear leukocytes were modifications of assays described (Rainard, 1985; Rajkovic and Williams, 1985). A strain of E.coli obtained from a clinical case of mastitis was incubated overnight in BHI at 38°C, centrifuged at 1000 g, washed 2x and adjusted to 10^7 CFU/ml in DPBS (by spectrophotometric measurement of bacterial cell concentration at 620 nm). For the assay, 40 μl of PMNL suspension were incubated with 20 μl of E.coli suspension and 40 μl of 25% [v/v] serum pool in DPBS in round-bottom microtiter plates. After 2 h at 38.5 or 42°C on a plate rotator (plates at 75° from horizontal), 20 μl of [methyl-^{3}H] thymidine (0.2 μCi) in DPBS were added with 100 μl of either DPBS (phagocytosis

assay) or 0.2% SDS in DPBS (killing assay). Plates were incubated for another 60 min at 38.5°C to allow incorporation of [methyl-^{3}H] thymidine into DNA of noningested and/or surviving bacteria. Controls with no E.coli (background, B) and with no PMNL (100% incorporation, I) were incubated simultaneously. Bacteria were harvested onto glass fiber filters using a semiautomatic cell harvester. The incorporated radioactivity was determined by scintillation counting. For cells of each cow, test (T), B and I were set up as quadruplicates and averaged for calculation of the phagocytosis index (PI) and killing index (KI) as $\{1-[(dpm_T-dpm_B)/(dpm_I-dpm_B)]\}x100$ %.

Migration of PMNL was evaluated under agarose (Nelson et al, 1973). Approximately 5 ml agarose solution (1% [w/v]) containing 5% [v/v] bovine calf serum were poured into a plastic Petri dish of 6 cm diameter. Rows of 3 wells of 5 mm diameter were cut 3 mm apart into the approximately 2 mm thick agarose layer. Twenty-five microliter of PMNL suspension were pipetted into the central well of each row. Into the other wells were placed either 25 μl DPBS (random migration) or 25 μl activated serum obtained as supernatant from the opsonization of zymosan (total migration). Dishes were incubated in 5% CO_2 for 2 h, and migration was stopped by placing plates at 4°C. Average distance migrated by the 100 cells most distant to the center well was measured using a 10 x 10 mm graticule in the ocular and the 40x magnification lens

from a light microscope. One graticule unit corresponded to 25.5 μm. Results were averaged from triplicate determinations per sample. Chemotaxis was calculated as the difference between total and random migration.

Lymphocyte Proliferation Assay

One hundred μl of lymphocyte suspension were placed into flat bottom test wells of a sterile 96-well microtiter plate, followed by various doses of PHA, PWM, or ConA in a total volume of 150 μl modified RPMI-1640 per well. After 48 h of culture in a humidified 5% CO_2 environment at different temperatures, 50 μl of [methyl-^{3}H] thymidine (0.1 uCi/well) in modified RPMI-1640 were added. In separate wells for estimation of viability, 50 μl of modified RPMI-1640 were added instead of radiolabel. Cells were harvested onto glass fiber filters 12 h later and washed with deionized water using a semiautomatic cell harvester. Radioactivity incorporated into newly synthesized DNA was determined by scintillation spectrometry. Stimulation by mitogens was calculated as S=T-B, where T represented dpm from cells incubated with mitogen, and B the background dpm for incubation with 0 μg mitogen. All determinations of [methyl-^{3}H] thymidine uptake were performed in triplicate, and viabilities were estimated without replication. An index expressing the inhibition by elevated temperature of incorporation of [methyl-^{3}H] thymidine was calculated as $I=\{1-[(S_{42})/(S_{38.5})]\}*100\%$.

Statistical Analysis

Data management and analysis were performed using the General Linear Model Procedure of the Statistical Analysis System (1982). Analysis of variance models for in vitro experiments included cow, temperature treatment for assays of neutrophil function, or dose of mitogen for the lymphocyte proliferation assay. All effects were considered fixed.

Several models were applied for the in vivo experiments. If no observations were missing, data were analyzed in a model for switchback design (Brandt, 1938) including group, linear and quadratic effects of period, cow nested in group, and cow nested in linear and quadratic effects of period. The environmental treatment effect was represented by Group x $Period_{quadratic}$ and tested with Group x $Cow(Period_{quadratic})$. In vitro temperature treatments were added to the model as a subplot of a split plot design. For phagocytosis and killing of E.coli by PMNL, observations for the second period were missing. Data were therefore analyzed as a split-split plot in time design including in vivo environmental treatment, tested by cow(environment), and in vitro temperature treatment, and its interaction with environmental treatment, tested by temperature x cow(environment); period was included in the sub sub plot. For the lymphocyte proliferation assay, observations for the third period were missing. Data for first and second period were analyzed as multiple Latin squares with 2 cows, one from each treatment group, assigned

at random to one of four 2 x 2 Latin squares, with in vitro temperature treatment and dose of PHA as subplot and sub sub plot in a split split plot design. In all models for the in vivo experiment the effect of cow was considered random, while all other effects were considered fixed.

Results

In Vitro Experiments with Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes from 3 lactating heifers were incubated at four different incubation temperatures (Figure 2-1, top panel A). Cytochrome c reduction was lowest at room temperature (23°C) at all incubation times ($P < 0.01$). After 5 and 10 min of incubation, activity at 38.5°C and 42°C was equal and higher than at 35°C ($P < 0.01$), but after 30 min of incubation, cytochrome c reduction was higher at 38.5°C than at 35 ($P < 0.05$) and 42°C ($P < 0.05$).

In another experiment, PMNL from 5 lactating heifers were preincubated at 38.5 and 42°C for 1 h. Viability of cells was not affected by preincubation temperature (95% at 38.5°C and 90% at 42°C, SEM = 2%). After preincubation, cells from 42°C were moved to 38.5°C and allowed to adjust to that temperature for 10 min before addition of cytochrome c and opsonized zymosan. Cells preincubated at 42°C reduced less cytochrome c than cells preincubated at 38.5°C at all incubation times ($P < 0.01$)(Figure 2-1, bottom panel B).

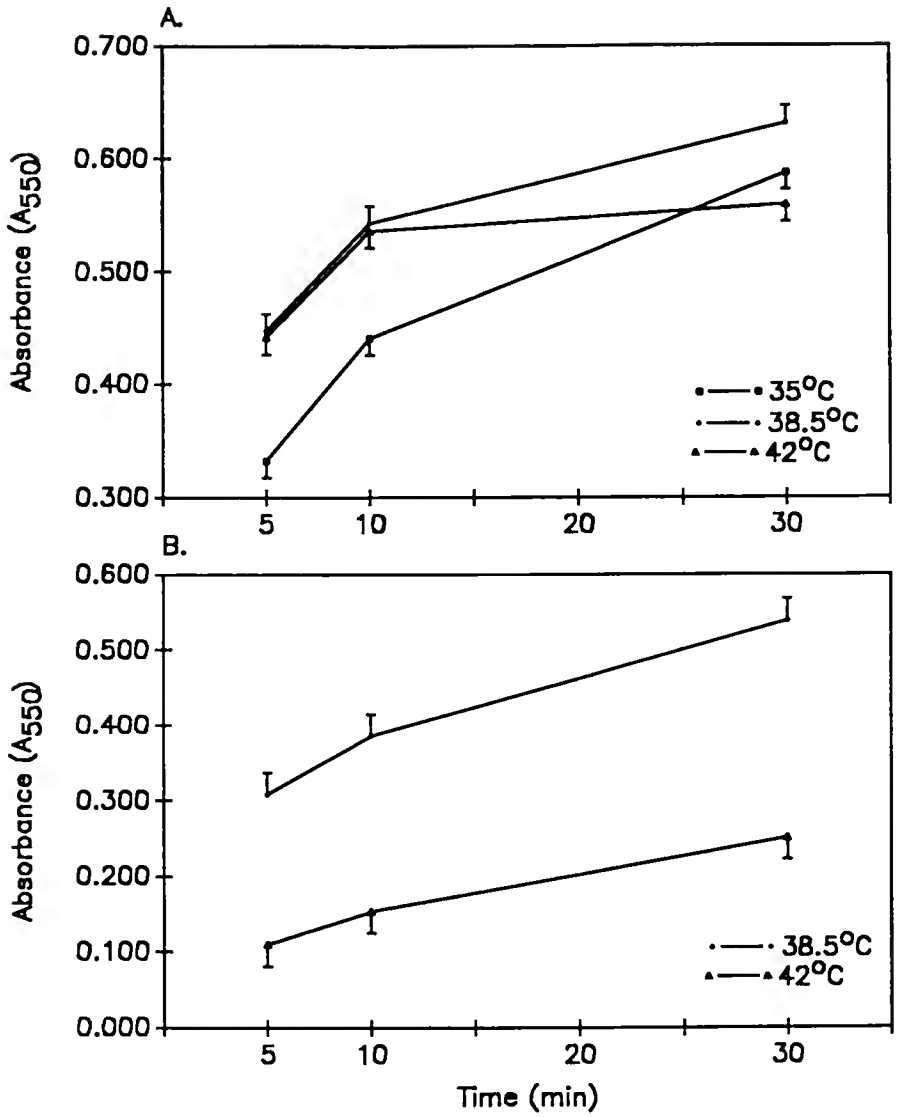

Figure 2-1. Panel A: cytochrome c reduction by PMNL obtained from 4 cows, as affected by incubation temperature. Least squares means and standard error bars. Cells were incubated at 4 different temperatures. At 23°C, A_{550} was 0.080, 0.176, and 0.432 after 5, 10 and 30 min of incubation; standard error of the mean was 0.015 (results not graphed). After 5 and 10 min of incubation, activity at 38.5 and 42°C was equal and higher than at 35°C ($P < 0.01$), but at 30 min incubation, cytochrome c reduction was higher at 38.5°C than at 35 ($P < 0.05$) and 42°C ($P < 0.05$). Panel B: cytochrome c reduction by PMNL obtained from 4 cows, as affected by pre-incubation temperature. Cells were preincubated at two different temperatures and then assayed at 38.5°C. Least squares means and standard error bars. Temperature affected cytochrome c reduction at all times.

To measure effects of temperature on phagocytosis and killing, PMNL from 4 cows were incubated with E.coli at 38.5 and 42°C. High temperature did not depress phagocytosis (PI: 86.0% at 38.5°C; 82.1% at 42°C; SEM = 2.5%), or killing (KI: 63.5% at 38.5°C; 58.8% at 42°C; SEM = 9.5%) of E.coli by PMNL. Viabilities of PMNL after 2 h of incubation with E.coli was not affected by incubation temperature (viability 94.5% at 38.5°C and 92.0% at 42°C, SEM = 0.8%, P = 0.11).

Migration of PMNL from 3 cows was evaluated at 38.5 and 42°C. Incubation at 42°C reduced random migration (99 vs. 54 μm, SEM=8 μm, $P < 0.01$), but did not affect chemotaxis (74 vs 71 μm, SEM=18 μm).

In Vitro Experiments With Lymphocytes

Lymphocytes from 4 cows were incubated with PHA (0 to 0.2 μg), or with PWM (0 to 2 μg) or ConA (0 to 2 μg) at 38.5 and 42°C. For all mitogens at all dosages, incorporation of [methyl-^{3}H]thymidine was reduced when lymphocytes were incubated at 42°C ($P < 0.01$) (Figure 2-2). There were no effects of dose of mitogen or dose x treatment for any mitogen.

In another experiment, lymphocytes from 3 cows were incubated with 0, 0.05, 0.1, and 0.2 μg PHA (Figure 2-3). The 60 hour incubation time was subdivided into three consecutive periods of 24, 24 and 12 hours. Eight temperature sequences were applied: 38.5-38.5-38.5, 38.5-38.5-42, 38.5-42-38.5, 38.5-42-42, 42-38.5-38.5, 42-38.5-42, 42-42-38.5, 42-42-42 °C

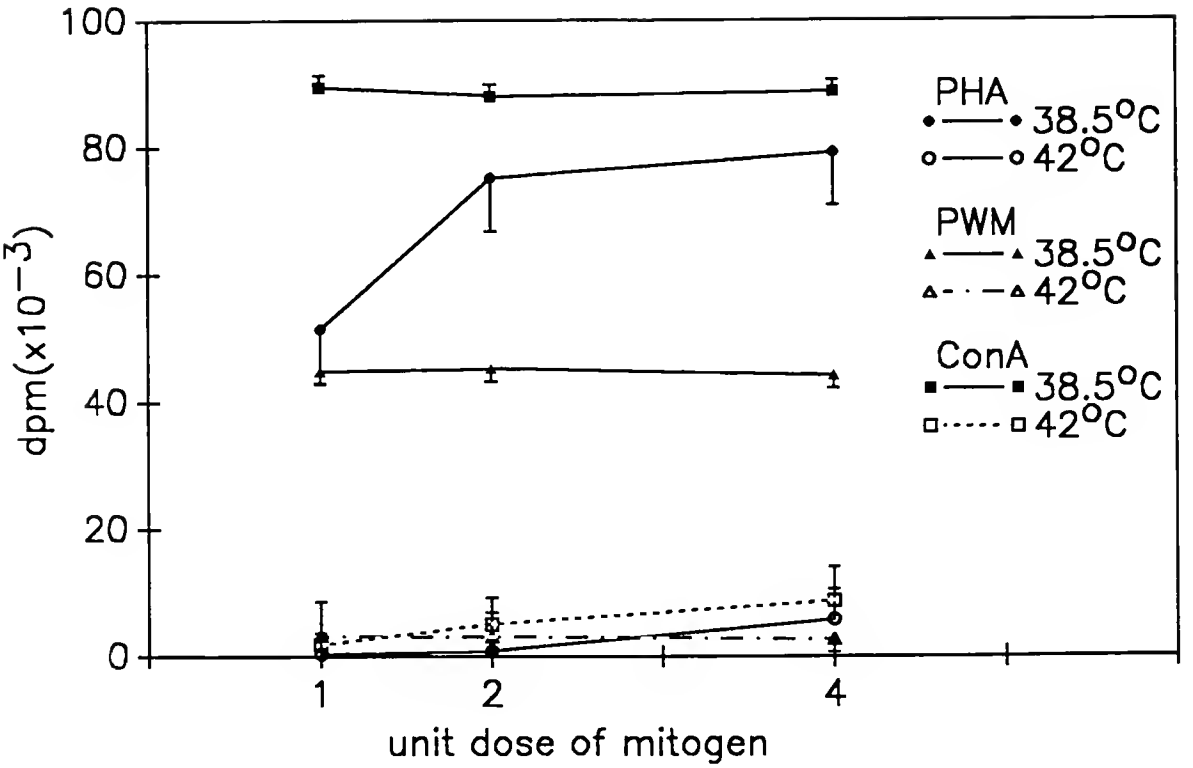

Figure 2-2. Proliferation of lymphocytes obtained from 4 cows, as affected by incubation temperature for 60 h, after stimulation with PHA, PWM, and ConA. One unit dose corresponds to 0.05 μg PHA/well, or 0.5μg PWM or ConA/well. Least squares means and standard error bars. Temperature affected proliferation at all dosages and mitogens ($P < 0.01$).

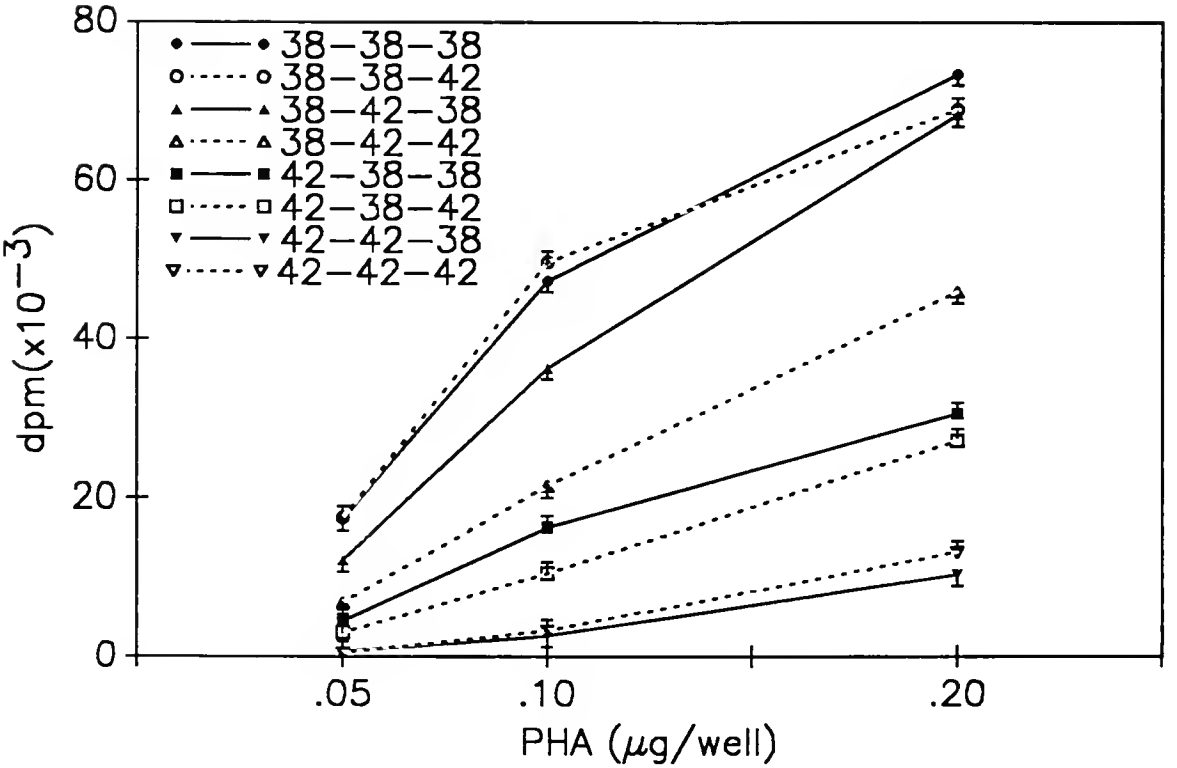

Figure 2-3. Proliferation of lymphocytes as affected by incubation temperature after stimulation with PHA. Cells obtained from 4 cows received temperature treatments during first 24 h, second 24 h, and last 12 h respectively of a 60 h culture (i.e. treatment with 42-38-38 indicated cells were cultured at 42°C for 24 h, then at 38.5°C for another 24 h, and at 38.5°C for the last 12 h). Least squares means and standard error bars.

(first 24, second 24, and last 12 h respectively). Temperature x dose affected [methyl-^{3}H]thymidine incorporation ($P < 0.01$). Incorporation was lowest for cells incubated at 42°C for 60 hours or for the first 48 hours (estimated contrast: $P < 0.01$). Incubation during the first or second 24 hour period at 42°C depressed [methyl-^{3}H]thymidine incorporation compared to 38.5°C, with incubation at high temperature during the first 24 h having the greatest effect, regardless of the subsequent temperatures (estimated contrast: $P < 0.01$). High temperature during the last 12 hour period had no effect on incorporation. Viabilities of lymphocytes evaluated for cells stimulated with 0.2 μg PHA were not different after incubation for 60 hours at 38.5 (58.3%) or 42°C (49.8%, SEM=3.82%, $P = 0.17$).

Effects of Heat Stress in Vivo

Rectal temperature (TR: 38.9°C; HS: 41.2°C; SEM=0.1°C, $P < 0.01$), respiration rate (TR: 60 breaths/min; HS: 114 breaths/min; SEM=4 breaths/min, $P < 0.01$), and heart rate (TR: 73/min; HS: 90/min; SEM=4/min, $P < 0.05$) were higher in HS cows. Rectal temperatures ranged from 37.9 to 39.9°C in TR and from 40.1 to 42.8°C in HS.

Total number of leukocytes in blood was higher in HS cows than in TR cows (Table 2-1). No differences were detected in relative amounts of each leukocyte type. Nineteen of 31 quarters were free of infection for the duration of the experiment. Staphylococcus spp. were recovered at least once

Table 2-1. Least squares means of peripheral blood leukocyte counts, and percentages of mononuclear cells, neutrophilic and eosinophilic polymorphonuclear leukocytes, and milk somatic cell counts for cows placed in thermoregulated and heat stress environment.

Environm. Treatment	Total Leukocytes (10^{-6}/ml)	Mononucl. Cells (%)	Neutroph. PMNL (%)	Eosinoph. PMNL (%)	Somatic Cells* [log(n x 10^{-3}/ml)]
Thermo-regulated	11.6[a]	66.9	27.1	5.9	2.023[c]
Heat Stress	14.0[b]	66.2	26.1	7.7	2.162[d]
SEM	1.2	3.8	2.7	1.5	0.051

Means with different superscripts are significantly different (ab: $P < 0.10$; cd: $P < 0.01$).

* Somatic cell counts in milk from uninfected and Staphylococcus spp. infected quarters were pooled.

Table 2-2. Least squares means for cytochrome c reduction, phagocytosis and killing of E.coli (upper panel), and migration (lower panel), by polymorphonuclear leukocytes, collected from cows placed in thermoregulated and heat stress environments,[a,b] and incubated at 38.5 and 42°C.

Environmental Treatment	Incubation Temperature (°C)	Cytochr.c Reduction (A_{550})	Phagocyt. of E.coli[c] (%)[d]	Killing of E.coli[c] (%)[d]
Thermoregulated	38.5	0.666	86.1	44.2
	42	0.529	81.7	44.2
Heat Stress	38.5	0.649	79.2	33.2
	42	0.524	70.3	36.6
SEM		0.008	2.8	2.2

Environmental Treatment	Incubation Temperature (°C)	Random Migration (μm)	Chemotaxis (μm)
Thermoregulated	38.5	24.5	77.3
	42	18.4	68.8
Heat Stress	38.5	37.7	35.4
	42	23.5	28.8
SEM		6.4	11.7

a: In vivo heat stress affected chemotaxis ($P = 0.07$).
b: Incubation temperature in vitro affected cytochrome c reduction ($P < 0.01$), phagocytosis of E.coli ($P = 0.06$), and random migration ($P = 0.05$).
c: Periods 1 and 3 only.
d: % inhibition of bacterial growth.

in 7 quarters from 5 cows, and Streptococcus uberis at least once in 5 quarters from 3 cows. Heat stress increased $\log_{10}$(SCC) (Table 2-1).

Assays for PMNL function were carried out at 38.5 and 42°C (Table 2-2). High incubation temperatures reduced cytochrome c reduction (A_{550}: 0.657 vs 0.527, SEM=0.006, $P < 0.01$), and PI (82.7% vs 76.0%, SEM=2.0%, $P = 0.06$), but did not affect KI (38.7 ± 1.75% vs 40.4 ± 1.4%). High incubation temperature also decreased random migration (31 μm vs 21 μm, SEM=3 μm, $P = 0.05$), while it did not alter chemotaxis (56 μm vs 49 μm, SEM=7 μm). There were no effects of in vivo heat stress on cytochrome c reduction, phagocytosis and killing of E.coli, and random migration, but chemotaxis was less for heat-stressed cows (TR: 73 μm; HS: 32 μm; SEM=12 μm, $P = 0.07$).

Lymphocytes collected from TR and HS cows were incubated for 60 h at 38.5 and 42°C after stimulation with 0.05, 0.1 and 0.2 ug PHA. High incubation temperature reduced ($P < 0.01$) the incorporation of [methyl-^{3}H]thymidine at all doses of PHA. The reduction in proliferation caused by culture at 42°C was less for cells obtained from cows during HS than from cells obtained during TR (environment x incubation temperature x dose of PHA: $P = 0.02$) (Figure 2-4). This alleviation of effects of elevated culture temperature occurred in period 1 (inhibition 60.7% vs 42.7%, SEM=5.0%). In period 2, incorporation of [methyl-^{3}H]thymidine was lower and the

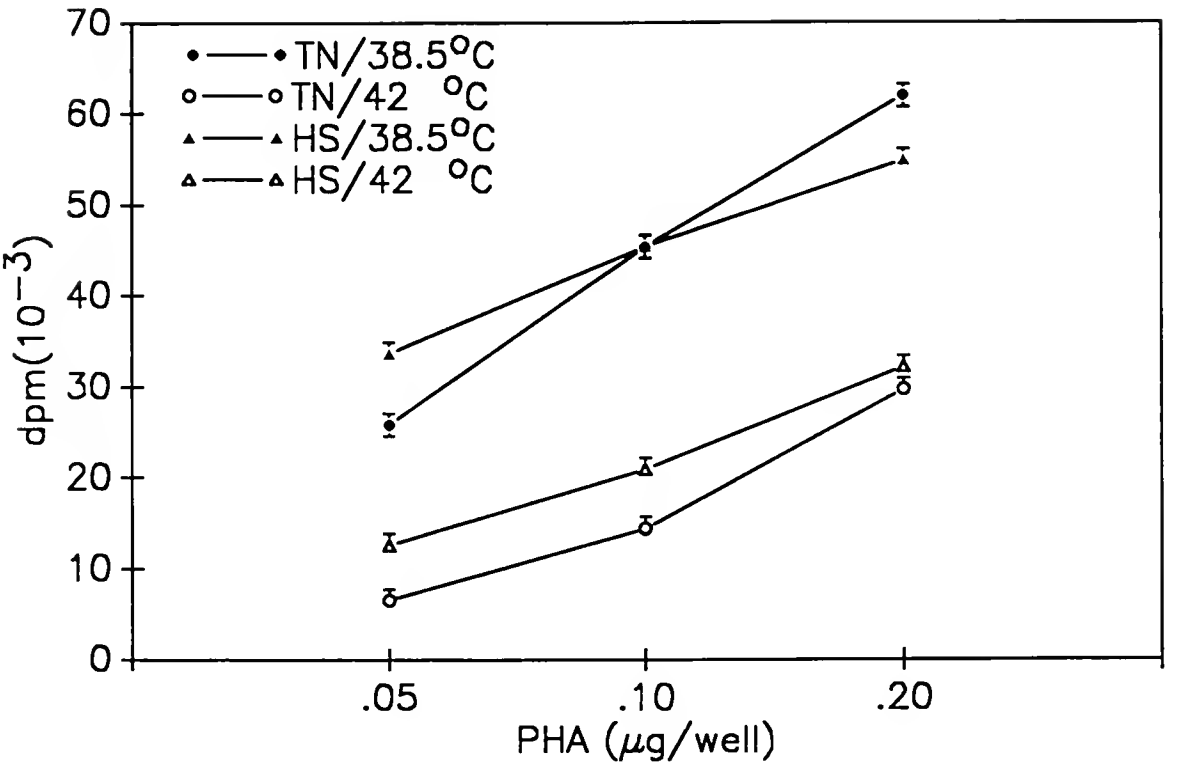

Figure 2-4. Proliferation of PHA stimulated lymphocytes at incubation temperatures of 38.5 and 42°C from cows maintained in a thermoregulated environment (TR) or in a heat-stress environment (HS). Least squares means and standard error bars. There was an environment x incubation temperature x dose of PHA interaction (P = 0.02).

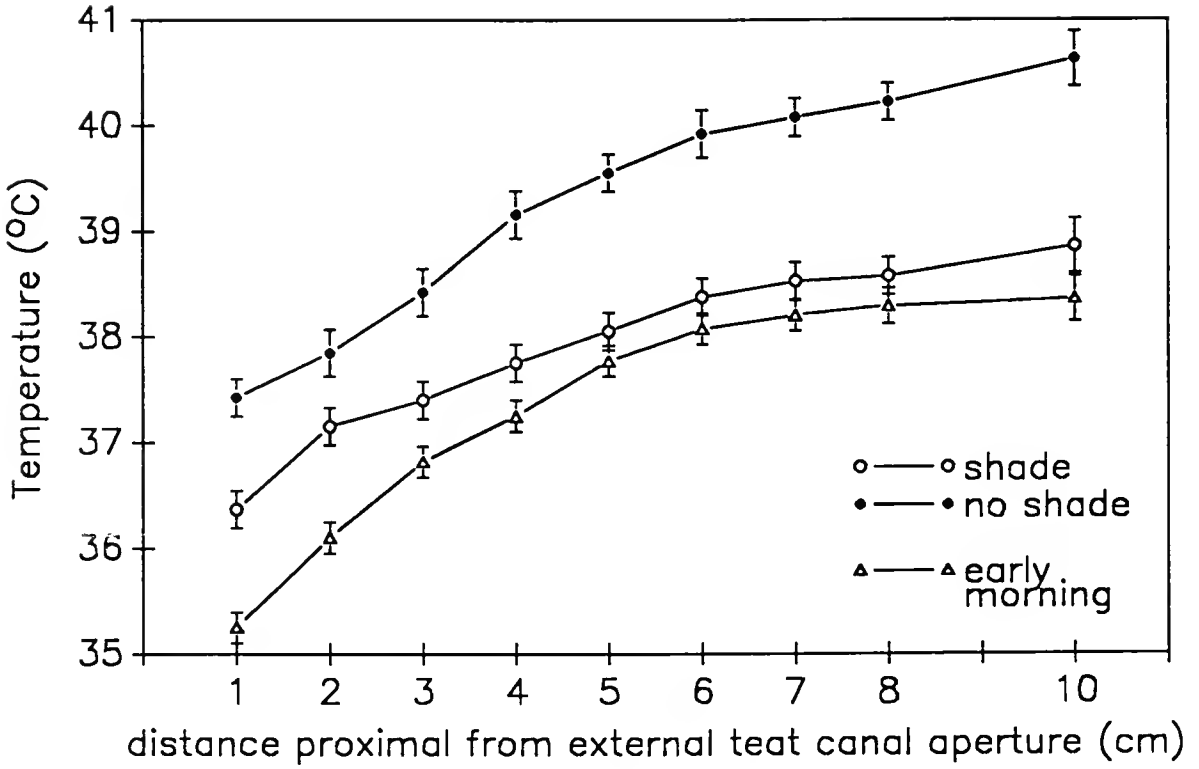

Figure 2-5. Intra-cisternal temperatures of cows submitted to different environments. Ambient temperature was 23°C at 8 a.m. and 33°C at 1 p.m. Rectal temperatures of cows were 38.9 ± 0.3°C in early morning. At 1 p.m. rectal temperatures were 39.1 ± 0.4°C for cows in shade and 40.8 ± 0.7°C for cows in no shade. Least squares means and standard errors. At 1 p.m., intra-cisternal temperature was affected by environment ($P < 0.01$), distance inserted ($P < 0.01$), and the environment x distance inserted interaction ($P < 0.01$).

of inhibition of incorporation were not different (86.1% vs 85.0%) for the two incubation temperatures (Environment x period: $P = 0.103$).

Intra-Cisternal Temperatures

At all times, intra-cisternal temperatures were lowest at a distance of 1 cm from the external teat orifice, and increased as insertion distance increased. Also, intra-cisternal temperatures reached body core temperature only when inserted 10 cm into the mammary gland cistern. Intra-cisternal temperatures were lowest at 8 a.m., when ambient temperature was 23°C, and rectal temperature was 38.9°C. At 1 p.m., ambient temperature was 33°C and rectal temperature averaged 39.1°C for cows in shade and 40.8°C for cows with no access to shade. Intra-cisternal temperatures were higher for cows with no access to shade (39.25 ± 0.07°C) than for cows in shade (37.90 ± 0.06°C; $P < 0.01$). In both groups, temperature increased as probe was inserted deeper ($P < 0.01$), and the increase was greater for cows in the no-shade environment (environment x distance inserted: $P < 0.01$; Figure 2-5).

Discussion

Results suggest that elevated temperatures can cause large effects on function of leukocytes in vitro, while they have more subtle effects in vivo. Lymphocytes were very sensitive to inhibition by high temperature in vitro, especially if elevated temperature exposure occurred in the first 24 h after stimulation. Inhibition due to elevated

temperatures was progressively less as exposure occurred later after addition of PHA. During the first 24 h of incubation, mitogen binding, signal transduction to stimulate mRNA production, interleukin 1 and interleukin 2 secretion, and interleukin 2 receptor expression occur, and may be hindered by elevated temperature. In humans increased temperature reduced biosynthesis of p35, an interluekin 1B precursor protein by peripheral blood adherent monocytes (Schmidt and Abdulla, 1988). It is also possible that cells became tolerant to heat as proliferation progresses because of the presence of heat shock proteins. Synthesis of heat shock proteins 70 and 90, for example, which likely protect cells from damaging effects of elevated temperature (Riabowol et al., 1988; Rose et al., 1989), is stimulated by mitogen activation (Haire et al., 1988).

High incubation temperatures inhibited certain functions of polymorphonuclear leukocytes. Random migration, but not chemotaxis was reduced, as was oxidative metabolism in response to stimulation with opsonized zymosan. Inhibition of oxidative metabolism by pre-incubation of cells at elevated temperatures indicates that a damage due to heat stress can persist. Increased production of oxygen-free radicals may be involved in cell injury by hyperthermia (Omar et al., 1987), and damage of PMNL by heat stress may be of a long term nature. Effects of elevated incubation temperature on phagocytosis and killing of bacteria were not as clear.

Phagocytosis was reduced at 42°C in one of two experiments, while effects of elevated temperature on killing were not observed in any experiment.

During the in vivo experiment, cows were submitted to severe heat stress, as reflected by the large increases in rectal temperature and respiration rates. Number of leukocytes in peripheral blood increased. Somatic cell counts in milk from heat-stressed cows were elevated, which might coincide with the numerical, though not significant elevation in random migration of polymorphonuclear leukocytes. It might also be that in heat-stressed cows, intra-cisternal temperatures are closer to normal body core temperatures, and migration of PMNL in the mammary gland was enhanced, because the cells were more active.

In spite of these differences in number of leukocytes in peripheral blood and of somatic cells in milk, leukocytes from heat-stressed cows generally functioned in vitro in a manner similar to that for leukocytes from control cows. There are several possible reasons for this. Cell function could be affected by procedures for preparation of cells for culture and existing differences may not be detectable. It is also possible that lymphocyte populations change in heat-stressed cows, as reportedly can occur in humans (Downing et al., 1988), or that they became resistant due to heat shock protein responses (Schlesinger, 1988; Welch et al., 1989). Some evidence that adaptation occurred in vivo is provided by the

fact that cells from heat-stressed cows in one period were less depressed by incubation at 42°C.

Results from measuring intra-cisternal temperatures indicate that the temperature at which PMNL function throughout much of the length of the teat is lower than body temperature. Results from in vitro experiments indicate that PMNL function was less at 35 and 23°C, than at 38.5°C. Thus, whole body hyperthermia could enhance function of PMNL in the teat cistern because of effects on intra-cisternal temperature. Nonetheless, PMNLs migrating to the mammary gland originate from the body core, it is not clear whether the potentially beneficial effect of heat stress on local function of PMNL would be counteracted by the fact that cells were heat stressed before entering the mammary gland.

To conclude, heat stress in vitro reduced the functional ability of PMNL and lymphocytes. This inhibition could not be shown in cells recovered from heat-stressed cows, although heat stress did alter certain properties of the immune system, including total number of circulating leukocytes and response of lymphocytes to heat stress. Although somatic cell counts were higher when cows were heat stressed, it remains to be determined whether a compromise in immune function caused by heat stress is responsible for increases in SCC in summer months (Paape et al., 1973; Wegner et al., 1976; Bodoh et al., 1976; Morse et al., 1988; Bray et al., 1989).

CHAPTER 3
ACTIONS OF BOVINE SOMATOTROPIN IN VITRO AND IN VIVO ON POLYMORPHONUCLEAR LEUKOCYTES AND LYMPHOCYTES IN CATTLE

Introduction

Physiological levels of bST in lactating cows are between 1 and 6 ng/ml plasma, while subcutaneous daily injections of 30 mg bST increase average concentrations to 10 - 20 ng/ml (Hart et al, 1985). While the effect of bST on milk yields is indirect, i.e., mediated through insulin-like growth factors, there is evidence for direct effects of somatotropin on cells of the immune system in the bovine and in other species. For example, the administration of exogenous somatotropin alters the function of the immune system in growth hormone-deficient animals. Depending on the type of deficiency and on species, administration of exogenous somatotropin enhances (Rovensky et al., 1982; Nagy et al., 1983; Davila et al., 1987), reduces (Rapaport et al., 1986, 1987), or has no effect (Blatt et al., 1987; Kiess et al., 1988) on the functional abilities of components of the immune system. Bovine and murine thymocytes have receptors for bovine somatotropin (Arrenbrecht, 1974), and receptors for human growth hormone have been detected on human lymphocytes (Lesniak et al., 1974; Kiess and Butenandt, 1985, 1987; Asakawa et al., 1986; Smal et al., 1987). Growth hormone production by rat and human lymphocytes has been

reported (Weigent et al., 1988). The activity of porcine blood derived mononuclear phagocytes was enhanced by administration of native and recombinant porcine somatotropin, as measured by an 18-fold increase in O_2^- production after stimulation with zymosan (Edwards et al., 1988). Superoxide production by neutrophils from dairy cows was significantly increased five to eight days after the start of bST treatment (Heyneman et al., 1989).

The objective of this study was to determine if bST alters the function of polymorphonuclear leukocytes and lymphocytes from heifers without somatotropin deficiency, when bST is given in vitro or in vivo.

Materials and Methods

Reagents

Recombinant bovine somatotropin for the in vitro experiment was Sometribove^R obtained from Monsanto Agricultural Company (St.Louis, MO). For the in vivo experiment, bST was obtained from American Cyanamid Company (Princeton, NJ). All other reagents were purchased as described in chapter 2.

Animals and Experimental Design

In vitro experiments were performed on leukocytes isolated from peripheral blood of lactating, primiparous Holstein cows. Effects of bST in vivo were evaluated using 24 heifers, randomly assigned to either medium or high growth rate groups. They were group-fed corn silage and grain

concentrate to achieve growth rates of less (medium growth) or greater (high growth) than 0.9 kg/d (high growth) from 4 to 12 mo of age. At 210 d of age, one half of the heifers in each growth rate group was assigned to one of 2 treatments [daily subcutaneous injections of 1 ml saline (placebo), or bST (12.6 mg/d in saline)] for 112 d.

Blood samples for the isolation of leukocytes for the investigation of components of the cellular immune system were collected between d 100 and d 112 of bST treatment by jugular veni-puncture, using evacuated heparinized blood collection tubes (Becton Dickinson, Rutherford, NY). Heifers were weighed bi-weekly. Three heifers from the placebo group were switched from the high growth rate group to the medium group because they gained less than 0.9 kg daily. Additionally, one heifer from the placebo group and one from the bST group were shifted from the medium to the high growth rate group because their daily growth rate was greater than 0.9 kg/d. Data were analyzed with 8 heifers in the medium growth/placebo group, 6 heifers in the medium growth/bST group, 4 heifers in the high growth/placebo group, and 6 heifers in the high growth/bST group.

Leukocyte Count, Differentiation and Isolation

Leukocyte count, and differentiation, and isolation of polymorphonuclear leukocytes and lymphocytes from peripheral blood of heifers were performed as described in chapter 2.

PMNL Functional Assays

Assays of cytochrome c reduction, phagocytosis and killing of E.coli, migration under agarose by polymorphonuclear leukocytes, and assay of proliferation of lymphocytes stimulated with mitogens were performed as described in chapter 2.

Statistical Analysis

Data management and analysis were performed using the General Linear Model Procedure of the Statistical Analysis System (1982). Least squares analysis of variance models for in vitro experiments included cow, bST and incubation temperature and their interactions. All effects were considered fixed.

For the in vivo experiment, least squares analysis of variance model was performed for a 2 x 2 factorial model. Growth rate and bST treatment were in the main plot and incubation temperature and dose of mitogen were in the subplots of a split-plot design. Effects in the main plot were tested with heifer(growth rate x bST). All effects were considered fixed, except for heifer, which was considered a random effect.

Results

In Vitro Effects of BST on PMNL

Polymorphonuclear leukocytes from 4 lactating primiparous cows were pre-incubated for 1 hour with 0, 10, 100, and 1000 ng bST/ml at 38.5 and 42°C. All tubes then were placed at

38.5°C for 10 min. Cytochrome c and zymosan were added and incubation continued for 30 min at 38.5°C. Viability of cells was not affected by concentration of bST (results not shown), but was higher at 38.5°C than at 42°C (92.4% vs 90.7%, SEM=0.3%; P = 0.04). Reduction of cytochrome c was less for PMNL pre-incubated at 42°C than for PMNL pre-incubated at 38.5°C (A_{550}: 0.506 vs 0.434, SEM=0.011; P < 0.01). Pre-incubation with bST did not affect subsequent cytochrome c reduction, and no pre-incubation temperature x bST dose interaction was detected.

In Vitro Effects of BST on Lymphocytes

Lymphocytes from 3 lactating primiparous cows were cultured for 60 h at 38.5 or 42°C with no mitogen, 0.5 μg PHA/well, 2 μg PWM/well, or 2 μg ConA/well, and with 0, 10, 100, and 1000 ng bST/ml. [Methyl-^{3}H]thymidine uptake was evaluated during the last 12 h of culture. After incubation for 60 h, viability of cells was not affected by bST, but was lower for lymphocytes incubated at 42°C (PHA: 70.3% vs 52.8%, SEM=1.2%, P = 0.01; ConA: 79.9% vs 68.9%, SEM=1.4%, P = 0.03). No temperature effects were detected for cells not stimulated with mitogen (69.6% vs 67.3%, SEM=3.1%), or stimulated with PWM (53.1% vs 59.2%, SEM=3.6%).

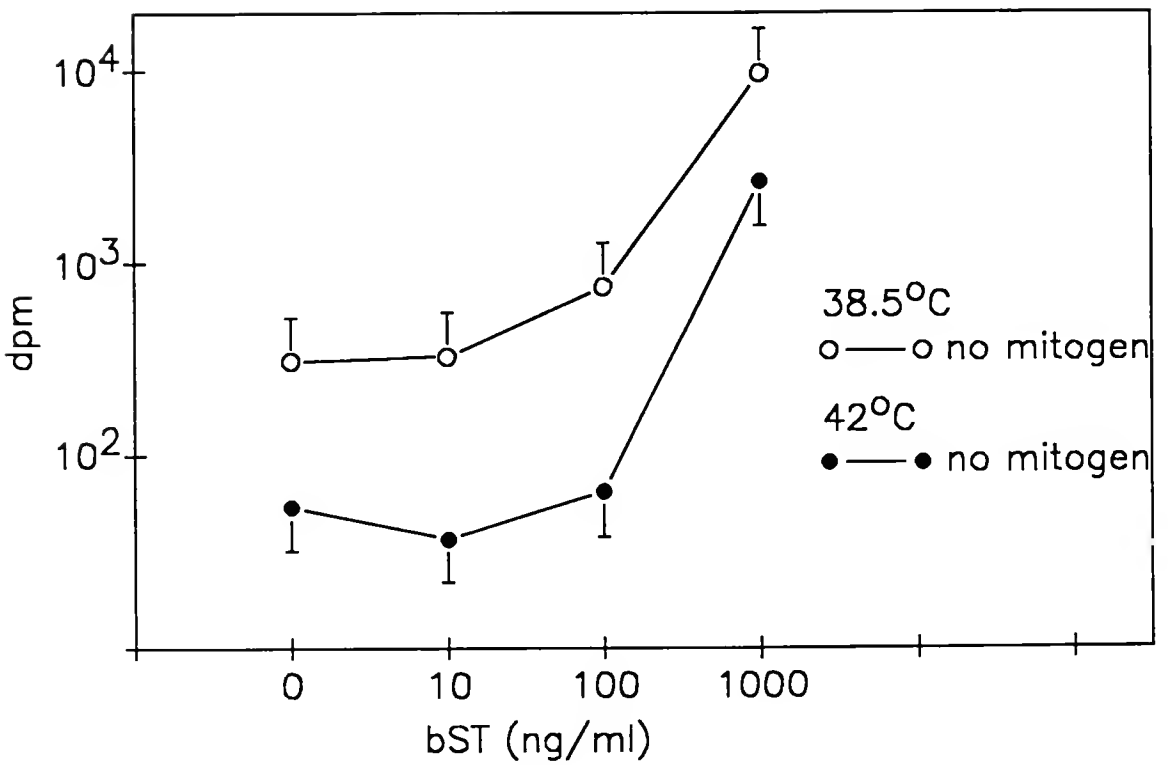

Figure 3-1. Incorporation of [methyl-^{3}H]thymidine by lymphocytes in the presence of 0, 10, 100, or 1000 ng bST/ml, and incubated at 38.5 and 42°C. Least squares means and standard error bars. Incorporation of [methyl-^{3}H]thymidine was depressed at all bST concentrations by incubation at 42°C ($P < 0.01$). Incorporation of [methyl-^{3}H]thymidine increased with increasing concentrations of bST ($P = 0.01$).

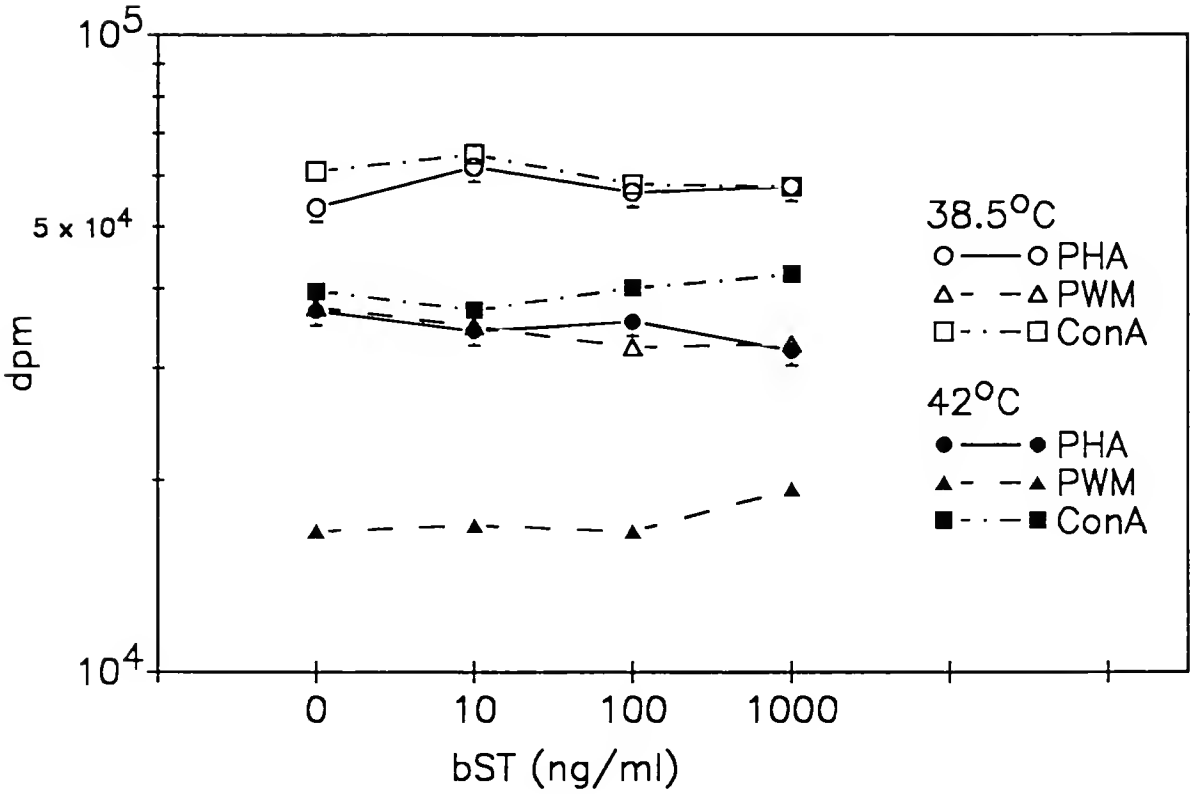

Figure 3-2. Incorporation of [methyl-^{3}H]thymidine by lymphocytes stimulated with 0.5 μg PHA, 2.0 μg PWM, or 2.0 μg ConA, in the presence of 0, 10, 100, or 1000 ng bST/ml, and incubated at 38.5 and 42°C. Least squares means and standard error bars. Incorporation of [methyl-^{3}H]thymidine was depressed by culture at 42°C for each mitogen at all concentrations of bST ($P < 0.01$). No effects of bST were detected.

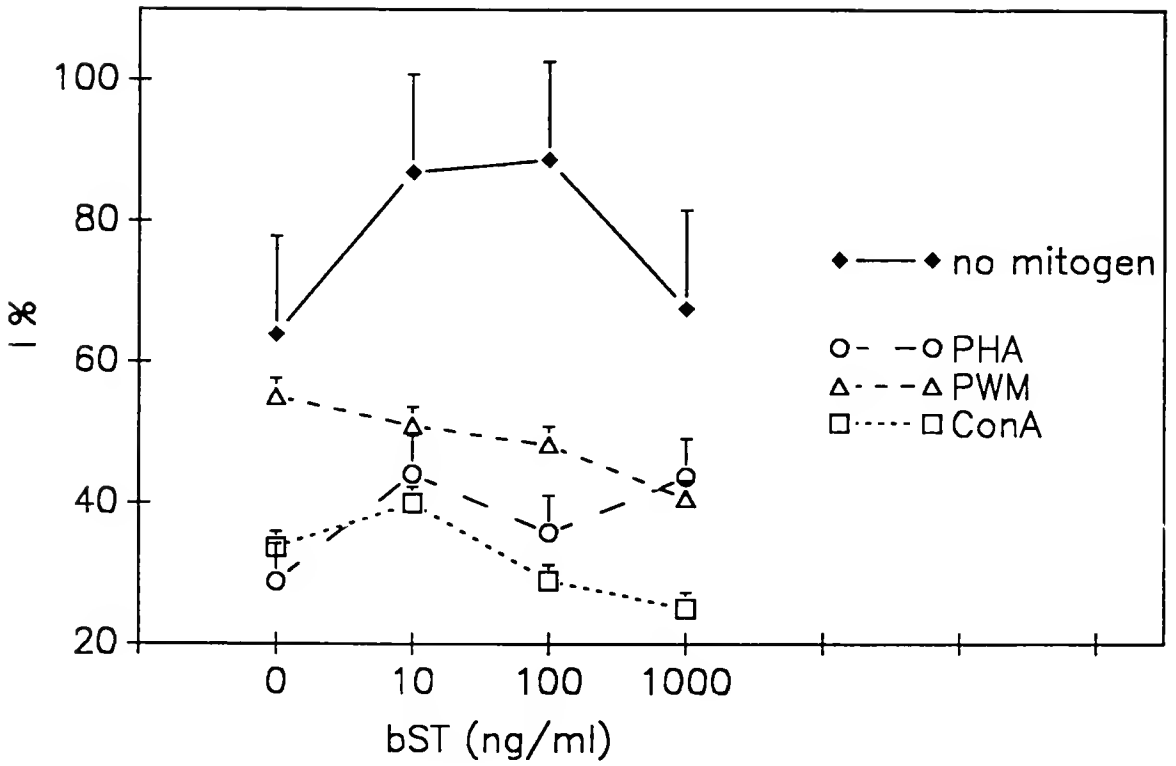

Figure 3-3. Inhibition of incorporation I% of [methyl-^{3}H]thymidine caused by culturing lymphocytes at 42°C vs 38.5°C for nonstimulated lymphocytes and lymphocytes stimulated with 0.5 μg PHA, 2.0 μg PWM, or 2.0 μg ConA, in the presence of 0, 10, 100, or 1000 ng bST/ml. Least squares means and standard error bars. No effects of bST on nonstimulated and PHA-stimulated cells were detected. With increasing concentrations, bST decreased inhibition of incorporation when lymphocytes were stimulated with PWM and ConA ($P < 0.05$).

Proliferation of lymphocytes was depressed by high temperature incubation at 42°C in nonstimulated and all mitogen stimulated cultures at all concentrations of bST (Figures 3-1 and 3-2). When cells were cultured without mitogen (Figure 3-1), bST had mitogenic effects at 1000 ng/ml (contrast 0, 10, 100 ng/ml vs 1000 ng bST/ml: $P < 0.10$). Incorporation of [methyl-^{3}H]thymidine increased with increasing concentrations of bST ($P < 0.01$). Treatment with bST had no effect on mitogen stimulated lymphocytes at 38.5 and 42°C (Figure 3-2). The index for inhibition of incorporation by elevated temperature, was not affected by bST, when cells were incubated without mitogen stimulation or with PHA. However, the inhibition of incorporation due to elevated temperature decreased with increasing concentrations of bST for cells stimulated with PWM ($P = 0.04$) and ConA ($P = 0.02$; Figure 3-3).

Effects of Growth Rate and BST in Vivo on White Blood Cell Counts

The total number of leukocytes ($P = 0.06$) and mononuclear cells ($P = 0.05$) were elevated in heifers treated with bST. The number of eosinophils ($P = 0.09$) was higher for heifers with high growth rate than for heifers with medium growth rate (Table 3-1).

TABLE 3-1. Number of total leukocytes, mononuclear cells, and neutrophilic and eosinophilic polymorphonuclear leukocytes from peripheral blood from heifers treated with placebo or bST, and fed to maintain medium and high growth rates.

Treatment	Total Leukocytes	Mononuclear Cells	Neutrophil PMNL	Eosinophil PMNL
	(cells/ml x 10^{-6})			
Placebo	15.6[a]	11.17[c]	4.10	0.57
bST	13.0[b]	8.99[d]	3.50	0.42
Growth rate				
medium	13.7	9.7	3.7	0.4[a]
high	14.9	10.4	3.9	0.6[b]
SEM	0.8	0.7	0.3	0.1

Means within a column with different superscripts differ. ab: $P < 0.10$; cd: $P < 0.05$.

Effects of Growth Rate and BST in Vivo on PMNL Function in Vitro

PMNL were incubated with opsonized zymosan for 30 min at 38.5 and 42°C. Cytochrome c reduction was reduced for cells incubated at 42°C (A_{550}: 0.595 vs 0.471, SEM=0.009; $P < 0.01$) for heifers in all treatment groups. No effects of growth rate, bST, or any interactions on cytochrome c reduction were measured (Table 3-2).

Elevated incubation temperature reduced phagocytosis (77.5% vs 71.5%, SEM=1.2%; $P < 0.01$) and killing (50.8% vs 41.4%, SEM=2.4%; $P < 0.01$) of E.coli by PMNL. Phagocytosis (70.6% vs 78.5%, SEM=2.7%; $P = 0.06$) and killing (36.3% vs 55.9%, SEM=6.9%; $P = 0.06$) were improved in the high growth rate heifers, but bST had no effects on phagocytosis or killing (Table 3-2).

High incubation temperature reduced random migration (72.3 μm vs 42.4 μm, SEM=6.4 μm; $P < 0.01$). Random migration and chemotaxis were not affected by growth rate or by bST (Table 3-2).

Effects of Growth Rate and BST in Vivo on Lymphocyte Proliferation in Vitro

Lymphocytes were isolated from 16 heifers and incubated for a total of 60 h at 38.5 or 42°C after stimulation with 0, 0.05, 0.1, or 0.2 μg PHA/well (Figure 3-4). Incorporation of [methyl-^{3}H]thymidine by lymphocytes increased with concentration of PHA ($P < 0.01$) and was reduced at high incubation temperature ($P < 0.01$). Incorporation increased

TABLE 3-2. Cytochrome c reduction, phagocytosis (PI) and killing index (KI), random migration and chemotaxis of polymorphonuclear leukocytes isolated from peripheral blood of heifers treated with placebo or bST, and fed to maintain medium or high growth rates.

Treatment	Cytochrome c Reduction (A550)	PI (%)*	KI (%)*	Random Migration (μm)	Chemotaxis (μm)
Placebo	0.562	74.1	51.5	70.4	87.2
bST	0.504	75.0	40.2	44.1	92.8
Growth Rate					
Medium	0.501	70.6[a]	36.3[a]	45.4	60.9
High	0.564	78.5[b]	55.9[b]	69.4	119.1
SEM	0.074	2.8	7.0	16.6	27.0

Means within a column with different superscripts differ. ab: $P < 0.10$).

* % inhibition of E.coli growth.

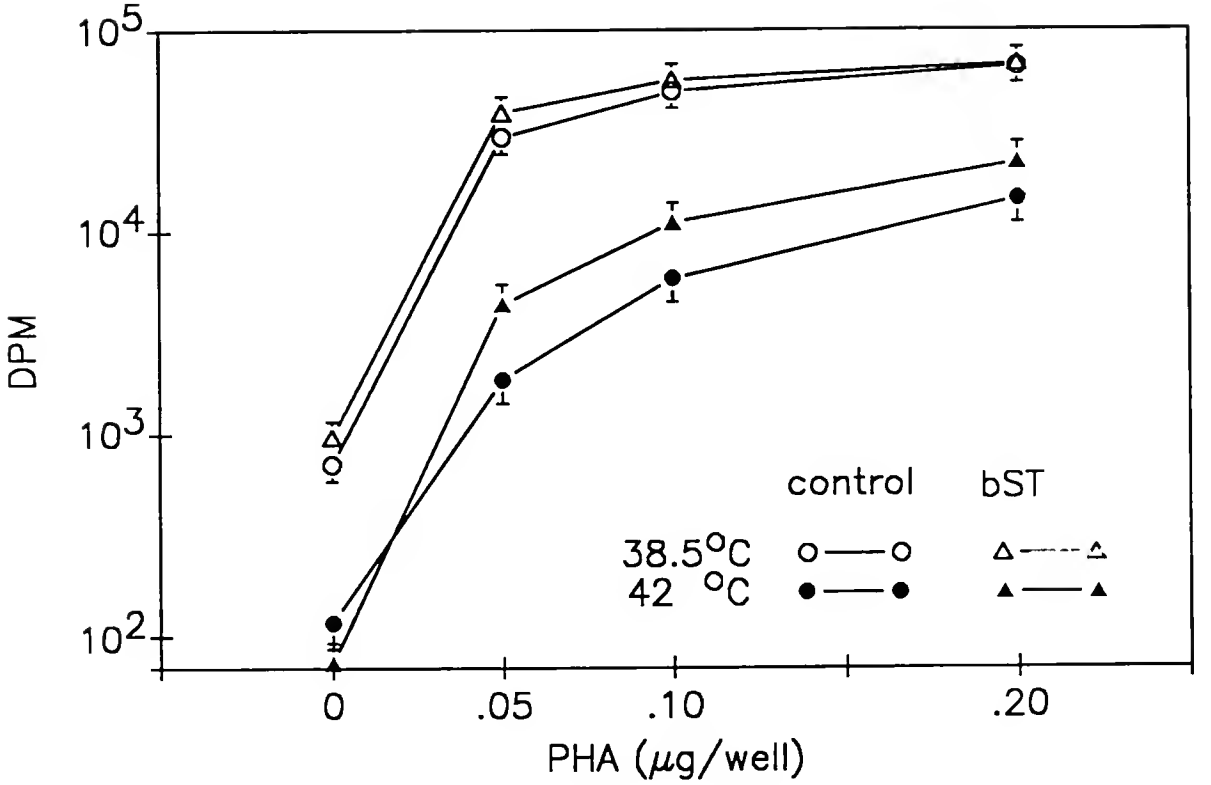

Figure 3-4. Incorporation of [methyl-^{3}H]thymidine for lymphocytes obtained from heifers treated with placebo or bST daily for 100 d and cultured in vitro at 38.5 and 42°C with 0, 0.05, 0.1, or 0.2 μg PHA. Least squares means and standard error bars. Elevated temperature depressed incorporation of [methyl-^{3}H]thymidine ($P < 0.01$), and depression of incorporation was less for cells obtained from bST-treated heifers ($P = 0.05$).

increased by high concentrations of PHA in lymphocytes incubated at low temperature (concentration of PHA x incubation temperature: $P < 0.01$). Depression of incorporation of [methyl-^{3}H]thymidine by elevated temperature was less for lymphocytes collected from bST-treated heifers than for lymphocytes collected from heifers treated with placebo (dose of PHA x incubation temperature x bST treatment: $P = 0.05$). This interaction was also apparent when an inhibition of incorporation index (%I) due to elevated temperature was calculated for each heifer. The %I decreased from 85.5 ± 1.5% at 0.05 μg PHA/well to 65.2 ± 1.5% at 2 μg PHA/well ($P < 0.01$) and was lower for cells collected from bST-treated heifers (68.8 ± 4.7%) than for cells from heifers treated with placebo (82.4 ± 5.4%; $P = 0.09$).

Discussion

Results indicate that bST alters lymphocyte function in vitro and in vivo while no effects on PMNL function were detected. Heyneman et al. (1989) also were unable to detect effects of bST in vitro on oxidative metabolism of bovine PMNL. However, they reported that bST treatment in vivo increased oxidative metabolism by PMNL from lactating cows 5 to 8 d after initiation of treatment. Differences in effects of bST may have resulted because animals used differed (growing heifer vs lactating cow), and so did the duration of bST treatment (100-112 d vs 5-8 d).

In contrast to the absence of effects of bST on PMNL, bST had dramatic effects on proliferation of lymphocytes in vitro, and more subtle effects on lymphocytes treated in vivo. At high concentration, bST itself was a mitogen, as indicated by the fact that 1000 ng bST/ml increased [methyl-^{3}H]thymidine uptake by cells without other mitogens to a level close to cells stimulated with phytohemagglutinin, pokeweed mitogen, or concanavalin A. Whether this effect is of physiological importance is not known. Concentrations that were mitogenic were much greater than found in blood of dairy cows, even when treated with bST (Hart et al., 1985; Lough et al., 1989; Zoa-Mboe et al., 1989). Somatotropin has been reported to be synthesized and released by lymphocytes (Weigent et al., 1988), and it is possible that local concentrations of bST may be high enough in certain lymphoid tissues to be mitogenic. The reason for culturing cells at 42°C was to create an immunodeficiency, as shown in chapter 2. The hypothesis was that bST would alleviate negative effects of elevated temperature. In the presence of phytohemagglutinin, pokeweed mitogen, or concanavalin A, bST did not increase [methyl-^{3}H]thymidine uptake at 38.5°C, but did reduce the inhibition of incorporation in cells stimulated with pokeweed mitogen and phytohemagglutinin caused by culture at 42°C. Lymphocytes from bST treated heifers also were less sensitive to the inhibiting effects of culture at 42°C. Bovine somatotropin thus conferred a degree of thermotolerance to lymphocytes.

In summary, bST in vitro and in vivo did not affect PMNL function, while having mitogenic and thermoprotective effects on lymphocytes. Physiological mechanisms and significance are unknown, but deserve further attention as bST may become available for use in dairy animals.

CHAPTER 4
INTERACTIONS OF HEAT STRESS AND ADMINISTRATION OF RECOMBINANT BOVINE SOMATOTROPIN ON PHYSIOLOGICAL AND IMMUNE FUNCTION OF LACTATING COWS

Introduction

The role of somatotropin during heat stress has not been completely resolved. Administration of bovine somatotropin increases metabolic rate in cows (Yousef and Johnson, 1966), and increases milk production (Bauman et al., 1989), which makes it difficult for cows in heat stress environments to maintain homeothermy. Not surprisingly, somatotropin secretion is reduced during heat stress (Mitra et al., 1972). With proposed use of bST to improve milk production also during summer (Mohammed and Johnson, 1985; Elvinger et al., 1988), it is important to understand the role of bST in physiological adjustments to heat stress.

There may be interactive effects between heat stress and somatotropin on immune function. Heat stress can decrease activity of the immune system both in vitro (chapter 2), and in vivo (Kelley et al., 1982b; Downing and Taylor, 1987; chapter 2), while bST can alleviate immuno-deficiencies (Nagy et al., 1983; Davila et al., 1987). Somatotropin countered cortisol-induced leukopenia in hypophysectomized rats (Chatterton et al., 1973), and enhanced antibody responses to

immunization in hypophysectomized rats treated with adrenocorticotropic hormone (Hayashida and Lee, 1957). Recently, bST has been reported to promote immune function (Edwards et al., 1988; Heyneman et al., 1989; chapter 3). Lymphoid cells express receptors for somatotropin (Arrenbrecht, 1974; Lesniak et al., 1974; Kiess and Butenandt, 1985, 1987; Smal et al., 1987), and Weigent et al. (1988) reported secretion of bST by lymphocytes.

It was the objective of this study to evaluate physiological effects of heat stress and bST, and to assess if in vivo bST treatment alleviates negative effects of in vivo heat stress on the immune system of lactating cows.

Materials and Methods

Reagents

Recombinantly-derived bovine somatotropin (bST) was Sometribove^{R} obtained from Monsanto Agricultural Company (St. Louis, MO).

Blood agar was prepared with Bacto Tryptose Blood Agar Base (dehydrated) from Difco Laboratories (Detroit, MI), and red blood cells obtained from cows. Bacto Brain Heart Infusion Broth, Triple Sugar Iron Agar, gelatin, and Motility Sulfide Medium were also from Difco Laboratories. McConkey Agar was purchased from BBL Microbiology Systems (Becton Dickinson & Co., Cockeysville, MD).

Mouse monoclonal antibodies (Table 4-1) to bovine leukocyte differentiation antigens were purchased from VMRD

Inc. (Pullman, WA). The fluorescein conjugated anti-mouse IgG was obtained from USB Corporation, Immunochemicals Division (Cleveland, OH). Mouse IgG_1, IgG_3, and IgM for isotype controls were from SIGMA Chemical Co. (St. Louis, MO).

Staphylococcus aureus vaccine, to be suspended in dextran sulphate for adjuvant, and antigen for enzyme-linked immunosorbant assay (ELISA), were donated by Dr. D.L. Watson, CSIRO, Armidale, New South Wales, Australia. Rabbit anti-bovine IgG_2 was from Nordic Immunological Laboratory (Capistrano Beach, CA). Goat anti-rabbit IgG (whole molecule) conjugated alkaline phosphatase and SIGMA 104 phosphatase substrate (p-nitrophenyl phosphate disodium) were from SIGMA Chemical Co. (St.Louis, MO). Alkaline phosphatase-conjugated affinity purified F(ab')2 fragment of rabbit anti-bovine IgG (F_c fragment specific) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Dulbecco's phosphate buffered saline solution (DPBS), Histopaque 1077, oyster glycogen, and p-nitrophenyl-N-acetyl glucosaminide were from SIGMA Chemical Co. (St.Louis, MO). Gamma-globulin free horse serum was purchased from GIBCO (Grand Island, NY) to be added at a concentration of 10% [v/v] to DPBS (DPBS+Ser).

Animals (Experiment A)

Thirty-four lactating Holstein cows were maintained in a thermoregulated environment, i.e., had access to shade and evaporative cooling facilities (sprinklers and fans). Sixteen

randomly chosen cows, assigned to the placebo group, received daily subcutaneous injections of 2.5 ml sodium bicarbonate; the other 18 cows received daily injections of 25 mg bST in 2.5 ml sodium bicarbonate. Treatment started on d 1 and lasted for 29 d. On d 10 of bST treatment, half the cows in each group were randomly assigned to one of two environmental treatment groups: cows either stayed in the thermoregulated (TR) environment or were placed in a heat-stress (HS) environment. In HS environment, cows were placed in a lot where they had no access to shade or to other cooling facilities. Environmental treatment lasted for 15 d, at which time heat-stressed cows were placed back into the thermoregulated environment.

On d -12, and on d 11 (d 2 of environmental treatment), all cows received an i.m. injection of 1 ml of Staphylococcus aureus vaccine in dextran sulphate solution. On d 19 (d 10 of environmental treatment), 10 ml of a 0.1% [w/v] oyster glycogen solution in DPBS were infused in one front quarter of 28 cows with low somatic cell counts (California Mastitis Test reaction negative).

Animals (Experiment B)

Seventeen cows, which in the previous experiment were not submitted to heat stress, and which had been treated with placebo or bST for 29 d, were maintained on sodium bicarbonate or bST treatment for 12 more d. On d 35 of bST treatment all 17 cows were exposed to heat stress environment for 7 d. One

cow in the placebo group became lame on d 3 of heat stress, and was removed from the data set.

Milk weights, Rectal Temperatures and Respiration Rates

For experiment A, milk weights were measured daily at the a.m. and p.m. milkings, starting on d -8. Rectal temperatures were measured between 2 and 3 p.m. on d 9, 10, 11, 13, 14, 15, 18, 19, 23, 24, 29 (d -1, 1, 2, 4, 5, 6, 9, 10, 14, 15 of environmental treatment). Respiration rates were measured at the same time on d 14, 19, 23, 24 (d 5, 10, 14, 15).

For experiment B, milk weights were measured daily at a.m. and p.m. milkings, starting 5 d prior to environmental treatment. Rectal temperatures and respiration rates were measured at 9.30 a.m., 11.00 a.m., and 12.30 p.m. on d 1 of environmental treatment, and daily between 12.00 and 1.00 p.m. thereafter.

Blood and Milk Samples

Samples were collected only for experiment A. Blood and milk samples were collected for determinations of anti-*Staphylococcus aureus* antigen in serum on d -12, and 18. Blood samples for peripheral blood cell counts and isolation of lymphocytes were collected on d 9, 10, 11, 15, 24 (d -1, 1, 2, 6, 15) and d 29. Milk samples for bacteriological evaluation were collected on d -14, -2, 6, 13, and 27. Milk samples for somatic cell counting and evaluation of N-acetyl-β-D-glucosaminidase were obtained on d 19, 20, 21, 22, 27 (d

0, .5, 1, 2, 3, and 8 post oyster glycogen infusion; d 10, 11, 12, 13, 18 of heat stress).

Analysis of Milk Samples

Foremilk samples for bacteriological evaluation were obtained aseptically. Ten μl of milk per quarter were spread on 1/4 blood agar plate and examined for bacterial growth after incubation for 24 and 48 h at 38°C. Bacterial growth was identified by evaluation of colony morphology, hemolysis pattern and gram staining. Coagulase test was performed on colonies of Staphylococcus spp. Colonies of Streptococcus spp. were tested for CAMP reaction and esculin hydrolysis. Intra-mammary infection was diagnosed when at least two consecutive samples were bacteriologically positive, except for the sample of d 27, where a single positive sample was sufficient for diagnosis of intra-mammary infection.

For the microscopic evaluation of somatic cell numbers, 10 μl of milk were expanded on a surface area of 1 cm^2 of a microscopic slide and stained with methylene blue. Twenty microscopic fields were counted using a 40x lens of a light microscope and somatic cell counts were calculated per ml milk (International Dairy Federation, 1981).

Levels of N-acetyl-β-D-glucosaminidase (NAGase) in milk were determined as described by Kitchen and Middleton (1975). Eighty μl of 5 mM p-nitrophenyl-N-acetyl glucosaminide in citrate buffer (pH 4.4) were added to 20 μl skim milk diluted 1:1 in citrate buffer and incubated for 30 min at 38°C. The

Table 4-1. Panel of mouse monoclonal antibodies to bovine leukocyte differentiation antigens.

Cell Line	Isotype	Antigen Specific
CH128A	IgG_1	BoT2 (CD2), anti-sheep red blood cell receptor
CACT83B	IgM	BoT4 (CD4)
CACT88C	IgG_3	BoT8 (CD8)
BAQ155A	IgG1	receptor on B-cells

reaction was stopped by the addition of 150 μl 0.5 M carbonate buffer (pH 10.3) and absorbance was read in a Microplate EL309 Autoreader photometer (Biotek Instruments, Winooski, VT) at 405 nm.

Blood Collection

Blood samples for the evaluation and isolation of leukocytes, and for measuring the packed cell volume, were obtained by veni-puncture, using evacuated heparinized blood collection tubes.

Peripheral Blood Leukocyte Count

Blood (20 μl) was diluted with 380 μl 3% [v/v] formic acid to lyse red blood cells (RBC). Leukocytes in the resulting suspension were counted in a hemocytometer.

Isolation of Mononuclear Cells From Peripheral Blood

Blood (10 ml) was diluted 1:3 in DPBS w/o Ca^{++} or Mg^{++}. The diluted blood was layered on 10 ml Histopaque 1077 and centrifuged for 30 min at 400 g. Cells in the interface between Histopaque 1077 and plasma were collected, washed twice in DPBS w/o Ca^{++} or Mg^{++}, and suspended in DPBS+Ser at 5 x 10^6 cells/ml.

Monoclonal Antibody Staining

Mononuclear cell suspensions (150 μl) were placed into wells of 96 well round bottom microtiter plates. All reagents were maintained at 4°C. Centrifugations and incubations were performed at 4°C. Plates were centrifuged at 350 x g for 3 min and supernatant discarded. Plates were gently vortexed to

loosen cell pellet. Fifty microliter of primary antibody (10 μg/ml DPBS+Ser) were added to appropriate wells (Table 4-1), plates were gently vortexed to mix cells and antibody prior to incubation for 15 min. Then, 150 μl DPBS+Ser were added to each well, the plates were gently vortexed, and then centrifuged at 350 x g for 3 min. Supernatant was discarded and the washing step repeated. Subsequently, 50 μl of second antibody (anti-mouse IgG from sheep, $F(ab')_2$ fragment, coupled to fluorescein isothyocyanate) at a dilution of 1:100 in DPBS+Ser, were added to each well, and plates were gently vortexed to mix cells and antibody. Mononuclear cells were then incubated for 15 min in the dark, before being washed twice with DPBS+Ser. The supernatant was discarded, and 40 μl of 1 % paraformaldehyde in saline solution [w/v] were added to each well. Plates were placed into the dark at 4°C until analysis. Controls were second antibody alone for background fluorescence, or appropriate isotype controls in place of primary antibodies. Immediately before analysis by flow cytometry, cells were washed twice with DPBS+Ser to remove excess paraformaldehyde solution. Time span between staining and flow cytometry did not exceed 10 h.

Flow Cytometry

A Facstar flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) was used for examination of cells, with a Consort 30 companion computer program for data acquisition and analysis. The data on 5000

cells selected for light scatter properties (forward and side scatter) were acquired in list mode. Cells were evaluated for fluorescence intensity. Cells with fluorescence intensity above intensity for background and isotype control were considered as positive, and their percentage was recorded. For B-cells, evaluation had to be altered, since no clear limits between background or isotype control fluorescence, and B-cell positive fluorescence could be detected. Thus one threshold value for fluorescence intensity for all samples was set, and cells staining more intensely were considered as B-cells.

Enzyme-Linked Immunosorbant Assay

Antibodies against staphylococcal surface antigens were measured in an enzyme-linked immunosorbant assay, as described by Watson and Davies (1985). Flatbottom, high-binding 96 well EIA/RIA plates (Costar, Cambridge, MA) were coated with 5 μg/ml antigen in coating buffer (0.05 M carbonate buffer, pH 9.6) for 1 hr at room temperature or overnight at 4°C. Dilutions of serum in incubation buffer (phosphate buffered saline solution-Tween 20, pH 7.4) were added. Wells were washed, and for measurement of antibodies of IgG isotype, alkaline phosphatase-conjugated rabbit anti-bovine IgG (1:500 in incubation buffer) was added. The plates were incubated for 1 h at room temperature, washed, and then incubated for 30 min with substrate (p-nitrophenyl phosphate in 0.1 M glycine solution, pH 10.4). For determination of antibodies of IgG_2

subtype, rabbit anti-bovine IgG_2 (1:500 in incubation buffer), alkaline phosphatase-conjugated goat anti-rabbit IgG, and substrate (p-nitrophenyl phosphate) were added sequentially. Plates were read in a Microplate EL309 Autoreader photometer (Biotek Instruments, Winooski, VT), and light absorbance at 405 nm after 30 min incubation at room temperature is reported.

Statistical Analysis

Data management and analysis were performed using the General Linear Model Procedure of the Statistical Analysis System (1982). Analysis of variance model was organized as a split-plot-in-time design, with parity (parity 1 and parity 2 or greater), environmental treatment, bST treatment, and interactions in the main plot tested by the cow within parity x environment x bST interaction. Day of treatment and interactions with main plot effects were in the sub-plot, tested by the overall mean square error.

Results

Physical Responses, Rectal Temperatures, Respiration Rates, and Packed Cell Volume

On d 10 (d 1 of environmental treatment) four cows from the heat stress/bST group became atactic in the late afternoon. Cow 1063 was removed from the trial. She was 8 years old and 217 d in lactation. Pre-environmental milk yield was 21.0 kg/d. Her rectal temperature on d 1 of environmental treatment had been 42.4°C. After falling down,

she suffered from a muscolo-scelettal dysfunction in her hind legs, and died two days later. Two quarters of the mammary gland were infected with Streptococcus uberis, one quarter with Streptococcus dysgalactiae, one quarter with Escherichia coli. On d 16 (d 5), cow 8740 collapsed and died within one hour. She was 56 d in her first lactation and produced 30.3 kg milk/d prior to initiation of heat stress. On d 1 of heat stress, her rectal temperature at 3.00 p.m. was 43.4°C, on d 5 it was 42.0°C. Two quarters were infected with Streptococcus dysgalactiae. Data of cows 1063 and 8740 up to the day of removal from the experiment were included in data analysis. Cow 8709 and 8712 also became atactic on d 1 of heat stress with rectal temperatures of 42.0°C, and pre-heat stress milk yields of 25.8 and 22.5 kg/d.

Rectal temperatures and respiration rates were measured throughout the environmental treatment periods (Tables 4-2, 4-3, 4-4; Figures 4-1, 4-2). For experiment A, rectal temperatures and respiration rates were higher in heat-stressed cows than in non heat-stressed cows ($P < 0.01$; Tables 4-2 and 4-3). Heat-stressed cows treated with bST had higher rectal temperatures than heat-stressed cows treated with placebo (environment x bST: $P = 0.01$; Figure 4-1). Packed cell volume in cows which were heat-stressed but not treated with bST, were higher than for cows in the other 3 groups ($P < 0.01$; Tables 4-2 and 4-3).

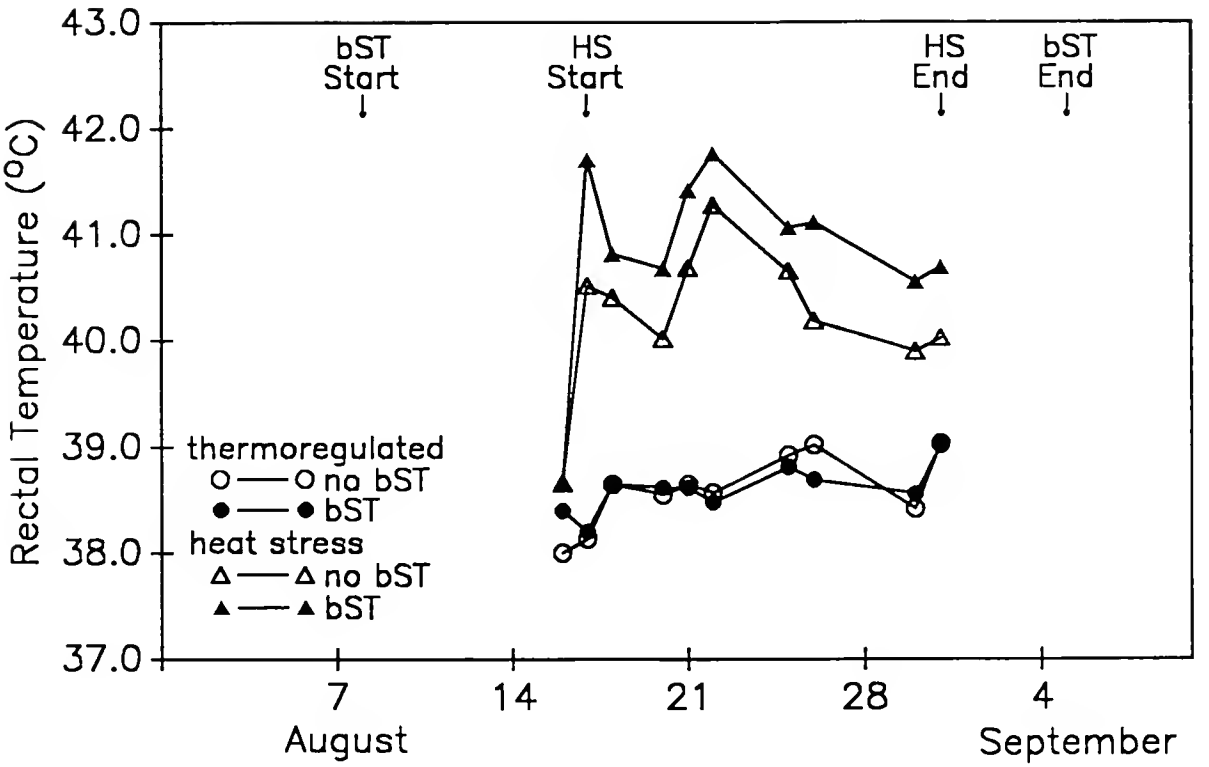

Figure 4-1. Rectal temperatures of cows treated with placebo or bST (25 mg/d) and exposed to a thermoregulated or heat-stress environment. During the bST/environmental treatment period, rectal temperatures were higher for cows in heat-stress environment ($P < 0.01$), and were higher for bST-treated cows ($P = 0.04$).

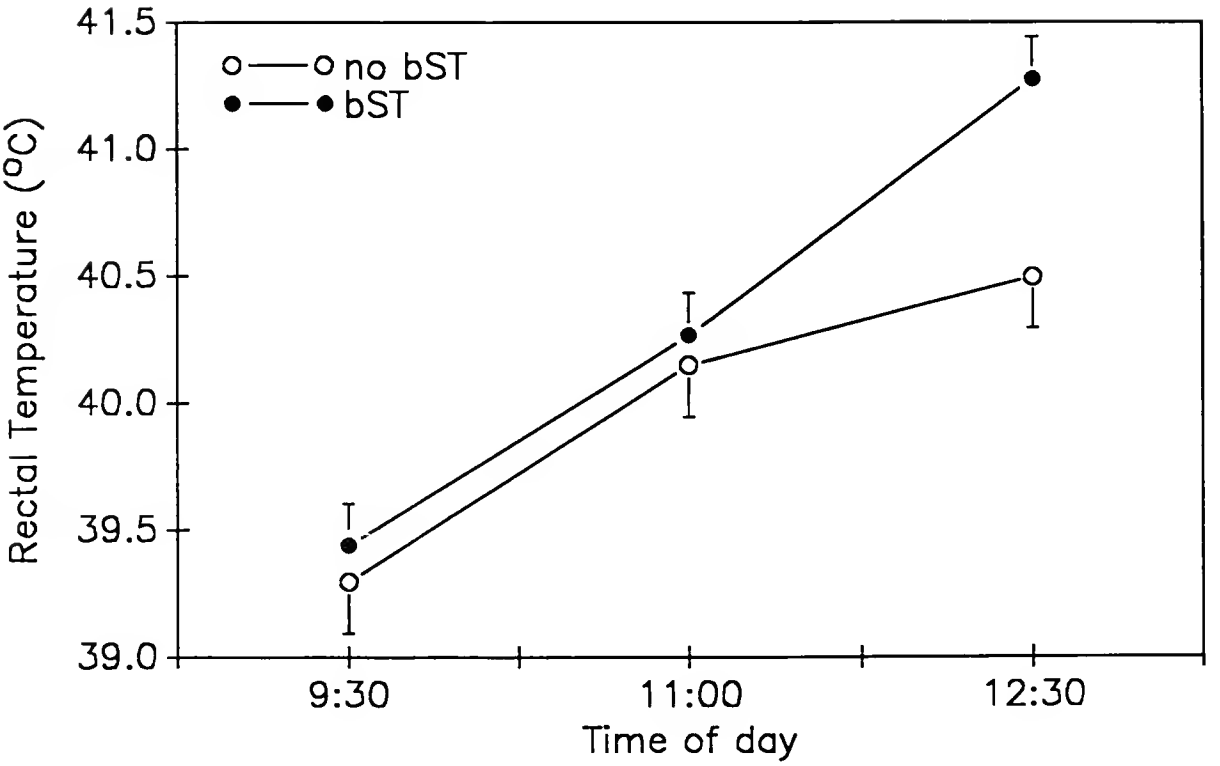

Figure 4-2. Rectal temperatures of cows treated with placebo or bST on the first day of exposure to heat-stress environment (Experiment B). Rectal temperatures tended to be higher in cows treated with bST (P = 0.09). Increase in rectal temperature is faster in bST-treated cows than in placebo-treated cows (bST x time: P = 0.4)

Table 4-2. Least squares means of milk yields, rectal temperatures, respiration rates, and packed cell volumes of cows treated with or without bST, and maintained in thermoregulated, and/or heat-stress environments.

Variable and Period	n[a]	Treatment Group				SEM
		TR/ placebo	TR/ bST	HS/ placebo	HS/ bST	
Milk Yield (kg/d)						
Pre-bST / pre-Environmental Treatment	192	22.4	23.4	25.1	22.8	1.3
bST / pre-Environmental Treatment	307	22.8	25.2	25.0	25.6	1.3
bST / Environmental Treatment	520	21.7	24.7	20.1	21.0	1.1
bST / Environmental Treatment (adjusted[b])		22.5	24.7	18.8	21.3	
Rectal Temperature[c] (°C)	293	38.6	38.7	40.5	41.1	0.1
Respiration Rate[c] (breaths/min)	97	68	59	116	113	5
Packed Cell Volume[c] (%)	97	33.0	33.8	35.4	32.6	0.6

a: number of observations for each variable.
b: adjusted for pre-bST/pre-environmental treatment period.
c: least squares means for bST/environmental treatment period.

Table 4-3. P-values for milk yields, rectal temperatures, respiration rates, and packed cell volumes of cows treated with placebo or bST, and maintained in thermoregulated, and/or heat stress environments.

Variable and Period	P-value		
	Environmental Treatment	bST	Interaction
Milk Yield			
Pre-bST / pre-Environmental Treatment	0.72	0.75	0.45
bST / pre-Environmental Treatment	0.67	0.24	0.79
bST / Environmental Treatment	0.01	0.10	0.75
bST / Environmental Treatment (adjusted[a])	**	**	0.09
Rectal Temperature[b](°C)	**	0.04	0.01
Respiration Rate[b](breaths/min)	**	0.57	0.65
Packed Cell Volume[b](%)	0.29	0.36	**

**: P < 0.01.
a: adjusted for pre-bST/pre-environmental treatment period.
b: P-values for bST/environmental treatment period.

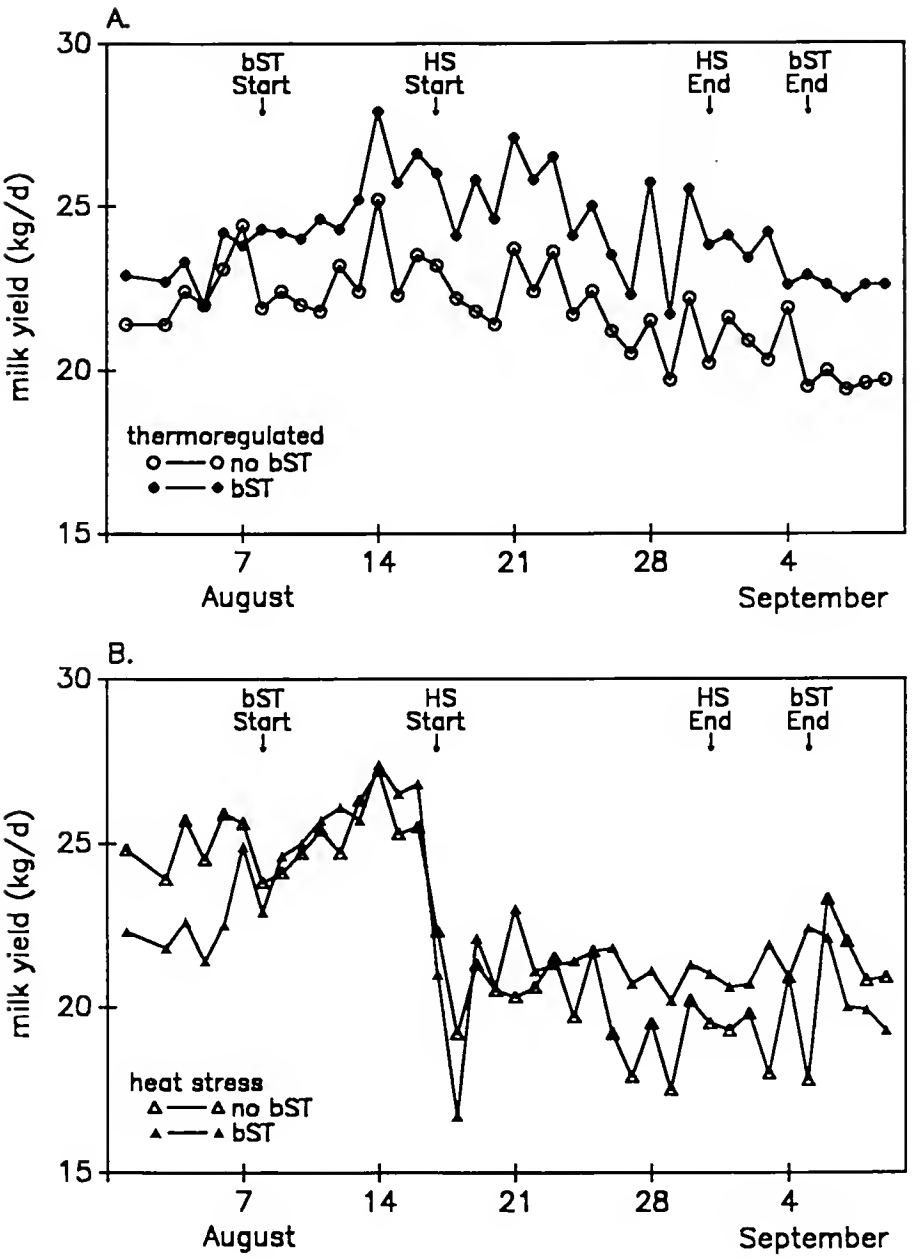

Figure 4-3. Milk yields of cows treated with placebo or bST (25 mg/d) and exposed to a thermoregulated or heat-stress environment. Panel A: cows maintained in thermoregulated environment during experimental treatment period. Panel B: cows maintained in heat stress environment during environmental treatment period. During the bST/environmental treatment period, milk yields were higher for cows in thermoregulated environment ($P = 0.01$), and tended to be higher for bST-treated cows ($P = 0.10$). When milk yields of the bST/environmental treatment period were adjusted for pre-bST milk yields, milk yields were higher for cows in thermoregulated environment ($P < 0.01$) and for bST-treated cows ($P < 0.01$).

Table 4-4. Least squares means of milk yields, rectal temperatures, and respiration rates of cows treated with placebo or bST, and maintained in heat-stress environment.

Variable and Period	n[a]	Treatment control	Treatment bST	SEM	P-value
Milk Yield (kg/d)					
Pre Heat Stress	76	21.1	22.4	1.1	0.44
Heat Stress	92	19.8	19.6	1.0	0.32
Heat Stress (adjusted[b])		19.9	19.4		0.50
Rectal Temperature[c,d] (°C)	91	40.8	41.1	0.1	0.10
Respiration Rate[c] (breaths/min)	111	127	122	3	0.45

a: number of observations for each variable.
b: adjusted for pre-heat-stress period.
c: least squares means for heat-stress period.
d: quadratic effect for milk yield as covariate: $P < 0.01$.

In experiment B, all cows were heat stressed. On d 1 of heat stress, rectal temperatures increased faster in bST-treated cows than in cows treated with placebo ($P = 0.04$; Figure 4-2). Over time, rectal temperatures tended to behigher in bST-treated cows than in cows treated with placebo ($P = 0.10$; Table 4-4), while respiration rates did not differ.

Milk Yields

In experiment A, milk yields were not different for treatment groups before bST treatment started (Tables 4-2 and 4-3; Figure 4-3). After initiation of bST treatment, milk yields increased in cows treated with bST. During the environmental treatment period, milk yields were lower in heat-stressed cows ($P < 0.01$), but heat-stressed cows treated with bST maintained higher milk yields than heat-stressed cows treated with placebo (after adjustment for pre-bST treatment yields, bST: $P < 0.01$; Environment x bST: $P < 0.10$; Tables 4-2, 4-3; Figure 4-3, Panel B).

In experiment B, milk yields were not different between bST and placebo cows during the period immediately preceding heat stress and during heat-stress period (Table 4-4).

Bacteriological Analysis of Milk Samples

Prior to the start of bST treatment, from 133 quarters, 6 quarters were infected with Staphylococcus spp., and 8 quarters with Streptococcus spp. During the bST-treatment period (including the environmental treatment period), none of 32 quarters in the TR/placebo group, four of 36 quarters in

the TR/bST group (1 Staphylococcus spp., 2 Streptococcus spp., 1 gram-negative), two of 31 quarters in the HS/placebo group (2 Staphylococcus spp.), and six of 34 quarters in the HS/bST group (cows 1063 and 8740) became infected. Of these infections, only two occurred in heat-stressed cows [one quarter in placebo cows (Staphylococcus spp.), and one quarter in bST cows (Streptococcus spp.)].

Somatic Cell Counts and Levels of N-Acetyl-β-D-Glucosaminidase in Milk

Peak somatic cell counts after oyster glycogen infusion were higher for cows in thermoregulated environment than for cows in heat stress environment (40.8×10^6 vs 17.1×10^6, SEM=3.9×10^6 somatic cells/ml; P = 0.01), and were 38.4×10^6, 43.5×10^6, 24.1×10^6, and 12.2×10^6 somatic cells/ml for TR/placebo, TR/bST, HS/placebo, and HS/bST cows respectively. Decrease of somatic cell counts was faster (environment x time post-infusion: P = 0.02; Figure 4-4). NAGase content was higher in multiparous cows (17.3 ± 1.5 nmoles/ml per min) than in primiparous cows (13.1 ± 1.0 nmoles/ml per min; $P < 0.05$). No effects of environment or bST on NAGase levels in milk were measured (Figure 4-5), although there is evidence that NAGase levels over time for placebo and bST groups were not parallel ($P < 0.01$). Pearson's coefficient of correlation for somatic cell counts and levels of NAGase was 0.58 ($n = 107$; $P < 0.01$).

Leukocyte Counts

Leukocyte counts in peripheral blood were generally unaffected by treatment, except that bST-treated cows had

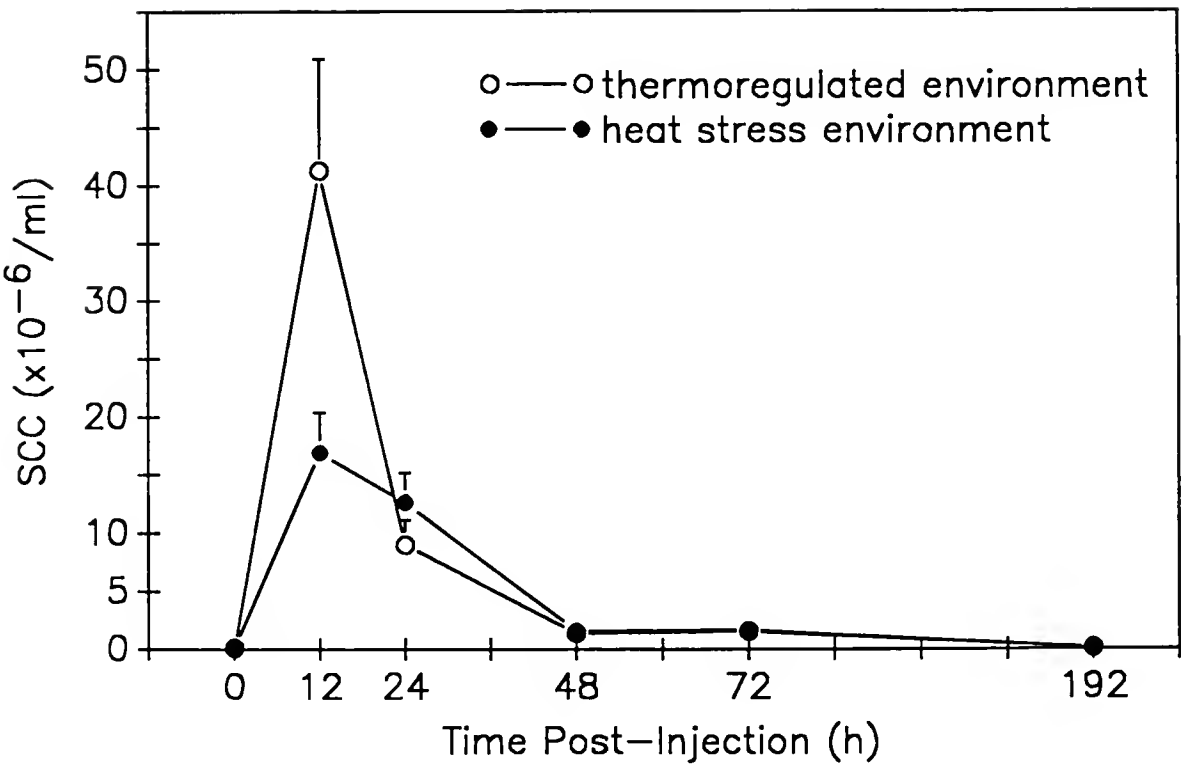

Figure 4-4. Somatic cell counts in milk from quarters infused with 10 ml of a 0.1% [w/v] oyster glycogen solution at time 0, from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Somatic cell counts from cows maintained in a thermoregulated environment increased to a higher level 12 h post-injection than from heat-stressed cows, and decrease to the base level was faster ($P < 0.10$). No differences due to bST treatment, or effect of interaction between environment and bST were calculated.

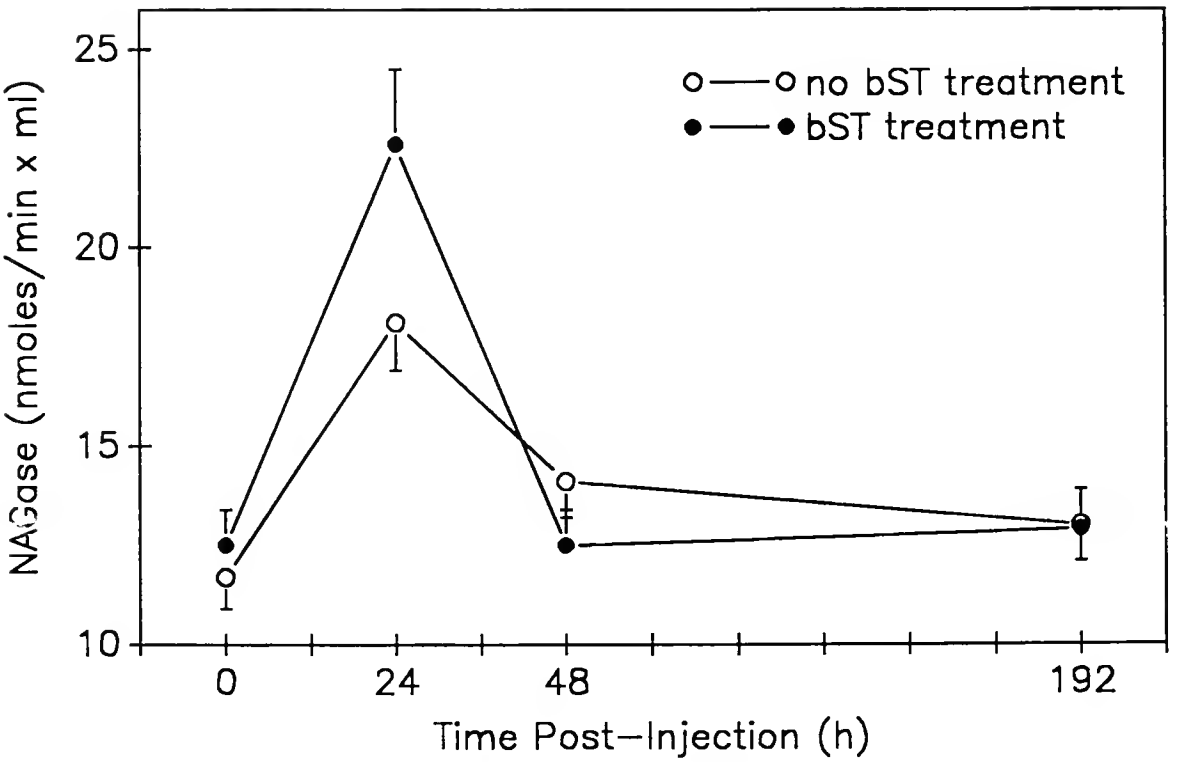

Figure 4-5. Concentration of N-acetyl-β-D-glucosaminidase (NAGase) in milk from quarters infused with 10 ml of a 0.1% [w/v] oyster glycogen solution at time 0, from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. NAGase decreased faster ($P < 0.10$) in bST-treated cows. No differences due to environmental treatment, or effects of interaction between environment and bST were calculated.

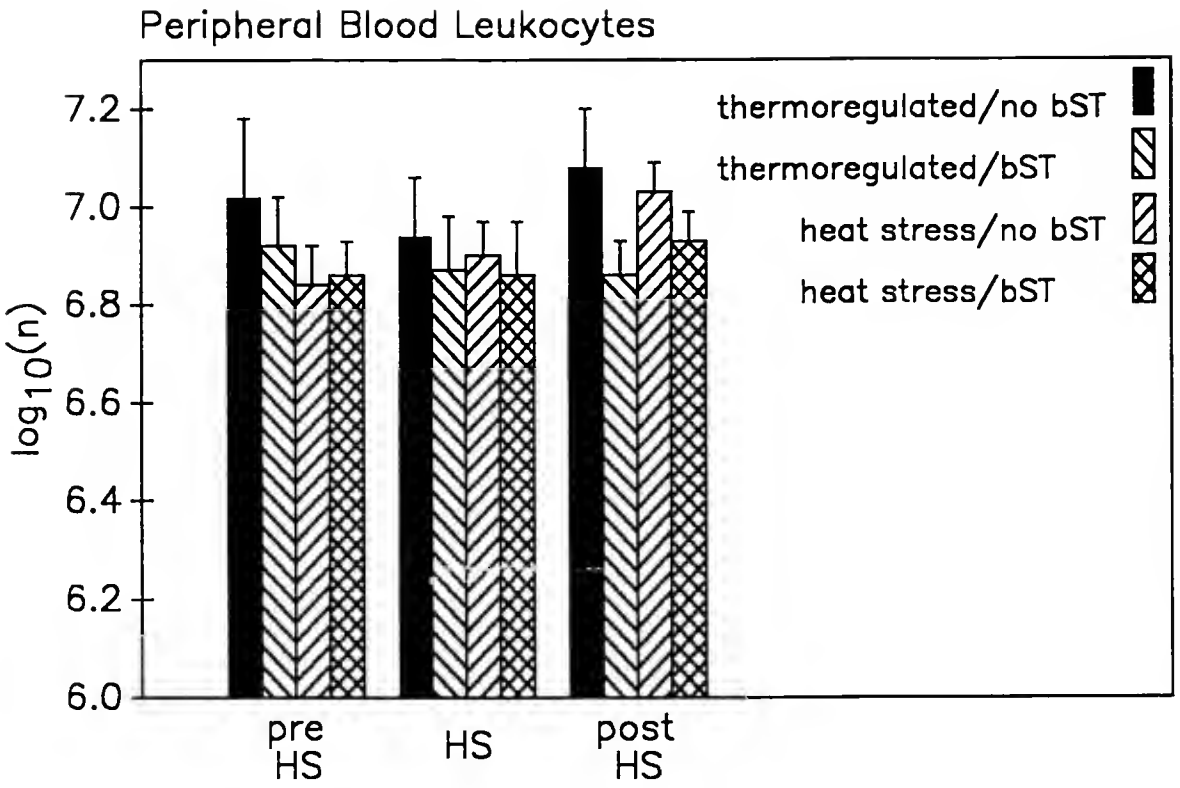

Figure 4-6. Number of leukocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences between treatment groups were detected in the pre-environmental and environmental treatment period, while leukocyte counts in bST-treated cows were lower than in placebo-treated cows in the post-environmental treatment period ($P = 0.06$).

lower counts than placebo cows after 29 d of treatment (P = 0.06; Figure 4-6).

Lymphocyte Populations and Subpopulations

No differences due to environmental or bST treatment, or to their interaction were detected for proportions of T-cells, $CD4^+$-cells, $CD8^+$-cells, or for the $CD4^+/CD8^+$-cell ratio (Figures 4-7, 4-8, 4-9, 4-10). Percentages of $CD2^+$-cells in peripheral blood ranged between 21 and 88%. The very low percentages were measured in cows with high leukocyte counts. No differences due to environmental or bST treatment, or to their interaction were measured for the three periods graphed in Figure 4-7. Percentages of $CD4^+$-cells were between 13 and 53%, percentages of $CD8^+$-cells were 7 to 47%. The higher $CD8^+$-cell percentages were measured in heat-stressed cows. The highest percentage of $CD8^+$-cells was measured in cow 8740 immediately pre exitus. The more severely heat-stressed cows also displayed $CD4^+/CD8^+$-cell ratio below 1.0 . $CD4^+/CD8^+$-cell ratio ranged from 0.5 to 4.8.

B-cell proportions ranged between 15 and 50 %. No differences due to environmental or bST treatment, or to their interaction were calculated (Figure 4-11).

Anti-Staphylococcus aureus antibodies

Serum samples collected before the first injection and after the second injection were analyzed for concentrations of IgG and IgG_2 specific for Staphylococcus aureus. Increase in

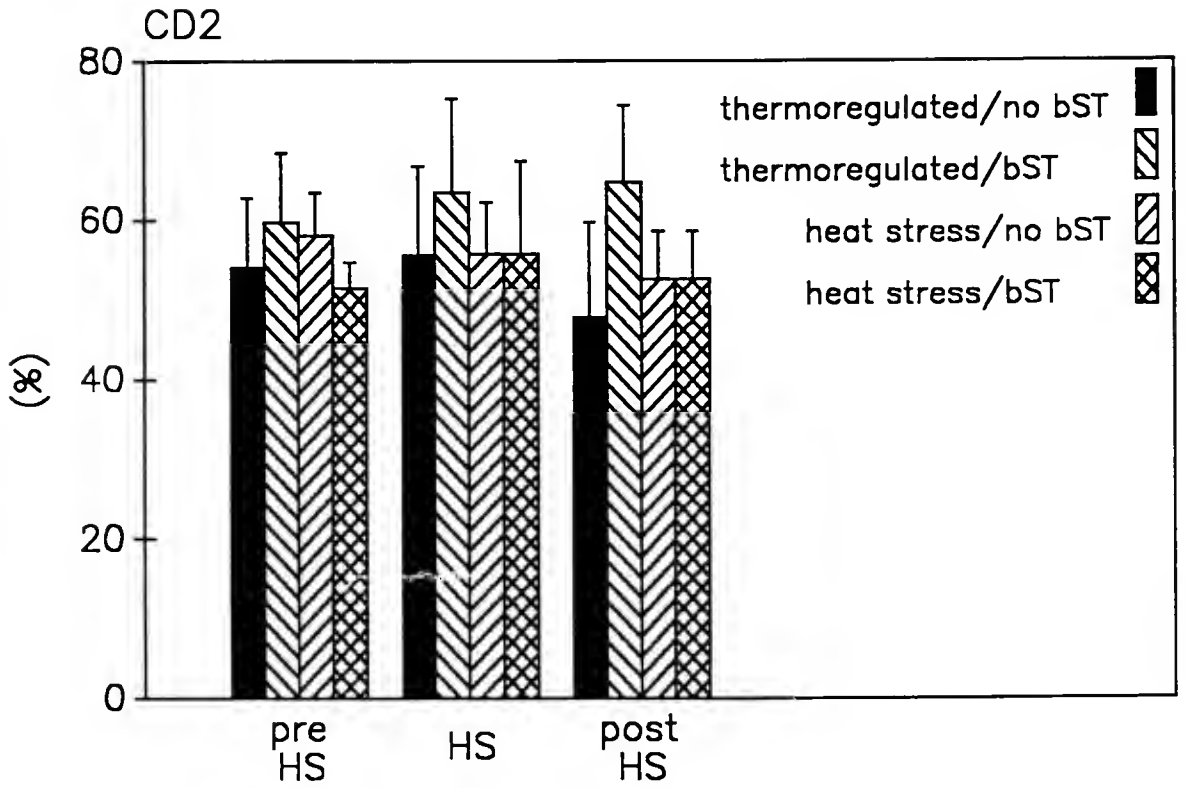

Figure 4-7. Percentages of $CD2^+$ lymphocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences were detected for treatment groups in all 3 periods.

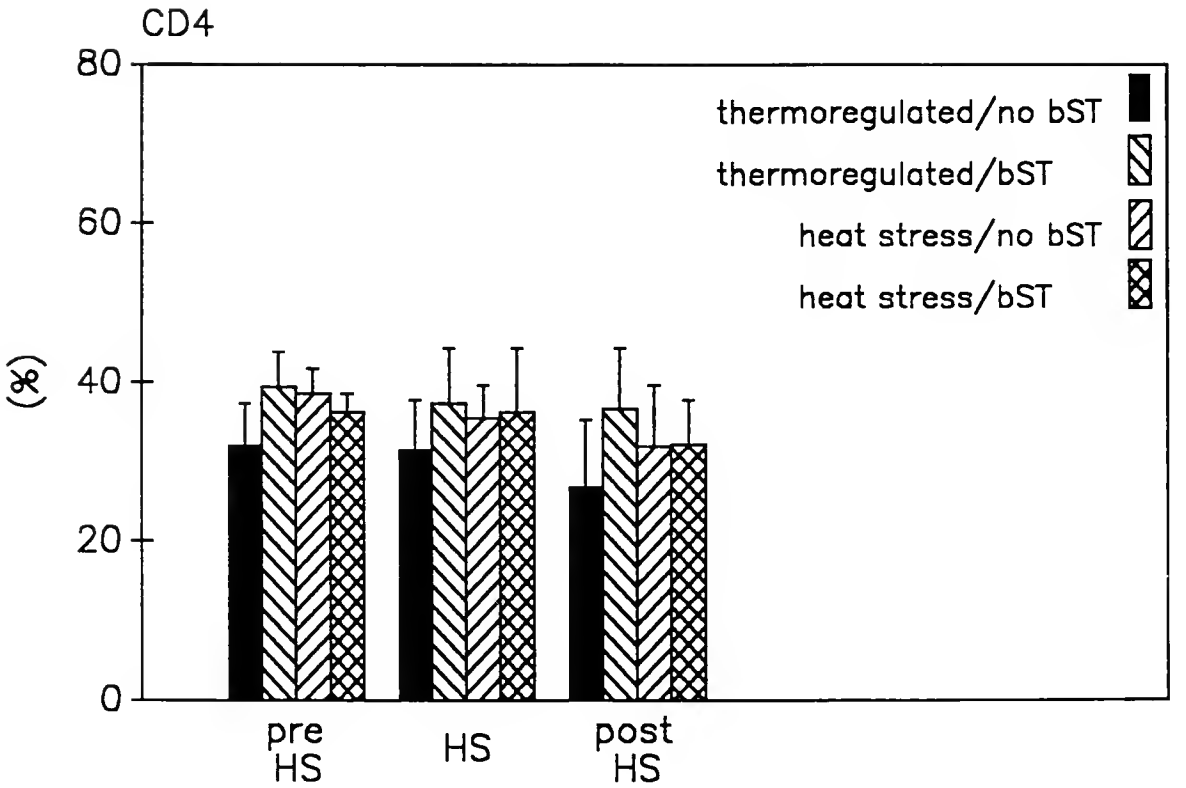

Figure 4-8. Percentages of $CD4^+$ lymphocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences were detected for treatment groups in all 3 periods.

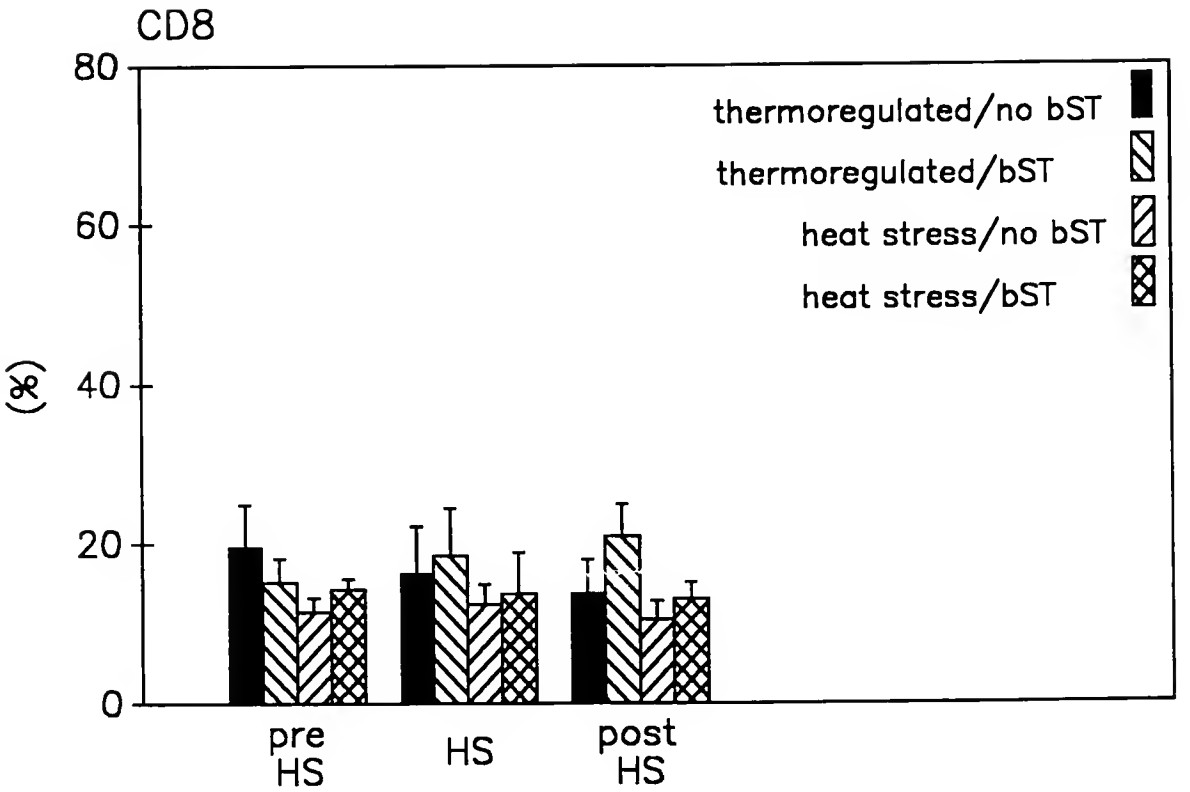

Figure 4-9. Percentages of $CD8^+$ lymphocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences were detected for treatment groups in all 3 periods.

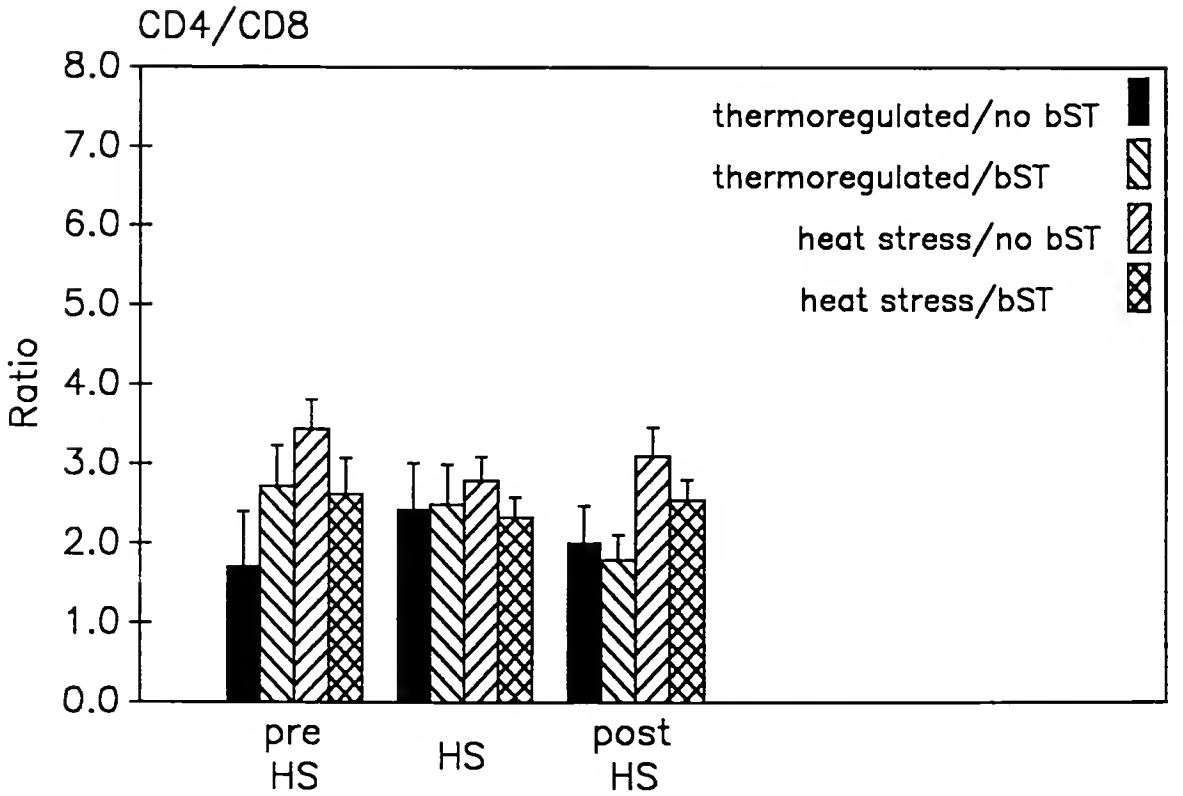

Figure 4-10. Ratio of $CD4^+/CD8^+$ lymphocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences were detected for treatment groups in all 3 periods.

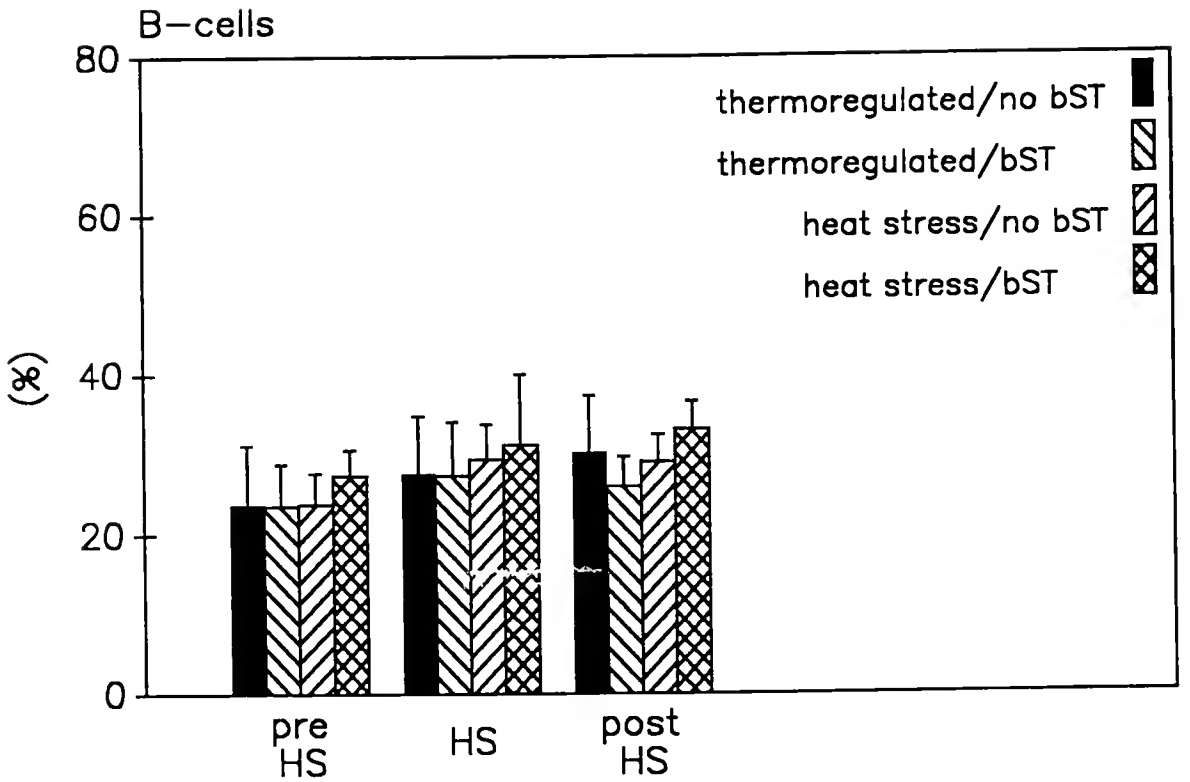

Figure 4-11. Percentages of B lymphocytes in peripheral blood from cows treated with placebo or bST and exposed to a thermoregulated or heat-stress environment. Bars represent least squares means (+ standard error) for treatment groups of cows sampled once in the pre-environmental treatment period, on d 1, 2, 6 and 15 of environmental treatment, and once 5 d post environmental treatment. No differences were detected for treatment groups in all 3 periods.

absorbance from samples measured at dilutions of 1:100 (IgG, A_{405} before: 0.847 ± 0.089; A_{405} after: 0.897 ± 0.085) and 1:250 (IgG_2, A_{405} before: 0.699 ± 0.073; A_{405} after: 0.722 ± 0.069) was very low. Absorbance at 405 nm for IgG and IgG_2 was not different between environmental treatment groups or bST-treatment groups, but A_{405} for IgG in samples from TR/bST cows was higher than for cows in TR/placebo cows, while A_{405} was not different between bST-treatment groups in the heat-stress environment (environment x bST: P = 0.05).

Discussion

Results indicate that cows treated with bST are affected more severely by heat stress than cows treated with placebo. Cows treated with bST still produced higher milk yields under heat stress environmental conditions than cows treated with placebo, as had been shown in other studies (Mohammed and Johnson, 1985; Elvinger et al, 1988). At the same time, rectal temperatures increased more in cows treated with bST. Yousef and Johnson (1966) found a 50 to 60 % increase in heat production in cows maintained at 38°C and injected with a single dose of 200 or 300 mg somatotropin, and increases in rectal temperatures, due to bST treatment, could be expected in heat-stressed cows, as had been measured by Mboe et al. (1989). Faster increase in rectal temperature within 3 h of heat stress, as measured in experiment B, could be an indicator for higher metabolic rate in bST-treated cows, but

also may be related to a decreased capacity to dissipate heat. Respiration rates were not affected by bST treatment.

In this study, packed cell volume was increased in heat-stress/placebo cows (Tables 4-2 and 4-3), although it did not increase in other heat-stress studies (Roman-Ponce et al., 1977; Schneider et al., 1984; Zoa-Mboe et al., 1989). Packed cell volume in heat-stress/bST cows was lower. Zoa-Mboe et al. (1989) related decreased hematocrit due to bST to increases in milk yield and expansion of plasma volume to support increased production of milk. Decreases in hematocrit due to bST treatment had been measured also by Soderholm et al. (1988) and Fullerton et al. (1989).

Environmental treatment did not affect leukocyte numbers in peripheral blood, while numbers of peripheral blood leukocytes were lower in cows treated for 29 d with bST. This latter finding corresponds to results reported in chapter 2, where bST reduced peripheral blood leukocyte counts in heifers treated for 100 d with bST. No effects of environment or bST on mean percentages of T and B lymphocytes, or on $CD4^+$- and $CD8^+$-cells, or on their ratio could be measured. Interestingly, $CD8^+$-cell proportions increased in severely heat-stressed cows, which corresponds to results in heat-stressed humans, where proportions of $CD8^+$-cells also were increased (Downing et al., 1988).

Heat stress depressed migration of leukocytes into the mammary gland after infusion of oyster glycogen. In chapter

2, random migration and chemotaxis by polymorphonuclear leukocytes were inhibited by culture temperatures of 42°C, and chemotaxis of polymorphonuclear leukocytes from heat-stressed cows was also depressed. This could explain increased incidence of mastitis in heat-stressed cows (Roman-Ponce et al., 1977; Morse et al., 1988), since leukocyte migration towards invading pathogens would be delayed. Treatment with bST did not alleviate heat-stress effects on somatic cell counts.

Overall, heat stress and bST had no major effects on tested components of the immune system. Cows treated with bST were more severely affected by heat stress than cows treated with placebo. The use of bST during summer in subtropical climate zones requires careful management to avoid overexposure of bST-treated cows to heat stress.

CHAPTER 5
ANALYSIS OF SOMATIC CELL COUNT DATA BY A PEAK EVALUATION ALGORITHM TO CALCULATE INCIDENCE RATES OF INFLAMMATION EVENTS

Introduction

Inflammation of the udder caused by a traumatic event, toxic agent, or invasion of microorganisms can be diagnosed through an increase in numbers of somatic cells in milk. While the somatic cell count (SCC) from an uninfected quarter averages 123,000 cells/ml (Mattila, 1985), the SCC in infected quarters is generally higher. Sheldrake et al. (1983) reported SCC between 84 and 832×10^3 cells/ml for quarters infected with minor pathogens, which include Corynebacterium bovis and coagulase negative staphylococci, and between 216 and $9,120 \times 10^3$ cells/ml milk for Staphylococcus aureus infected quarters. Ward and Schultz (1972) reported means of 770 to 2050×10^3 cells/ml milk in quarters infected with Streptococcus agalactiae and Streptococcus uberis.

Infection and inflammation resulting in an increase of SCC may affect one or more quarters (Natzke et al., 1972b). If composite milk samples are obtained from an udder inflamed in one quarter only, the dilution of the milk from that quarter by milk from the three healthy quarters is likely to mask the SCC increase in that quarter. This dilution effect is enhanced by the decreased milk yield of the inflamed

quarter and the concurrent compensating milk yield increase in the non-inflamed quarters (Woolford, 1985). A doubling of the total milk SCC, even at low SCC levels, may be the expression of an infection in one quarter (Natzke et al., 1972b).

Variability of SCC reported from individual cows over time results from 4 major sources: intra-assay variation of the somatic cell counting procedure (Schmidt-Madsen, 1975; Heeschen, 1975; Szijarto and Barnum, 1984; Miller et al., 1986), time of sampling and amount of milk in the udder (Convey et al., 1971; Duitschaever and Ashton, 1972; Fernando and Spahr, 1983), increasing age and stage of lactation (Bodoh et al., 1975; Sheldrake et al., 1983; Ali and Shook, 1983; Jones et al., 1984), and inflammation events in the mammary gland, most generally due to contamination by pathogens and infection (Rullmann and Trommsdorff, 1906). This latter source of variation is responsible for an increase in SCC of much greater magnitude than the other sources (Brolund, 1985).

An increase of SCC due to infection and inflammation is a sporadic event, of variable duration. The incidence of inflammation events (IE), as well as their severity can describe udder health and resistance of cows. The primary objective of this study was to develop a method to detect and evaluate IE. The method was to be independent from intra-assay variation, diurnal variations, and long term trends associated with lactation number and stage of lactation.

A peak evaluation algorithm used in endocrinology (PULSAR: Merriam and Wachter, 1982) was adapted to the evaluation of time series of SCC. Other objectives were to evaluate bST and seasonal effects on the incidence and severity of inflammation events in a lactating dairy cow population. Differences related to bST administration could be due to alterations of the cows' resistance status, while seasonal effects could be viewed either as an alteration of the immune status of the cows, or as a change of the challenge to the mammary gland by udder pathogens.

Materials and Methods

Animals and Data Collection

To evaluate effects of bST on SCC, 198 lactating Holstein cows on 2 Florida dairy farms were blocked by parity (parities 1 and 2+) and stage of lactation (SL, SL 1: d 61-100 post partum (p.p.); SL 2: d 101-140 p.p.; SL 3: d 141-180 p.p.) and were randomly assigned to 2 treatment groups. Cows in the bST-treatment group were injected bi-weekly with 500 mg Sometribove^{R} in a prolonged release system. Cows in the control group were not injected. Treatments were started on farm 1 on July 25 (n=72, treatment for 24 weeks) and October 17 (n=72, treatment for 14 weeks), and on farm 2 on July 19 (n=54, treatment for 20 weeks).

Milk yields were recorded and composite milk samples were collected at weekly intervals. Samples for somatic cell counting were analyzed by the Milk Quality Laboratory of the

Virginia Federation of Dairy Herd Improvement Association in Blacksburg, VA. Only cows with low SCC (n=171) prior to the start of treatment were used for the analysis.

For analysis, data were organized into three sets. Set A, the entire data set, represented 65 cows started in summer and 64 cows started in fall on farm 1, plus 42 cows started in summer on farm 2. Set B represented data from 65 cows on farm 1, started in summer, plus 42 cows on farm 2. Analysis of data sets A and B focused on effects of bST treatment. Set C was compiled to evaluate seasonal influences on IE, and contained data collected for 14 weeks from farm 1 (65 cows started in summer and 64 cows started in fall).

Least Squares Analysis of Variance

To normalize their distribution SCC data were transformed to a $\log_2$ scale. Least squares analysis of variance was used to evaluate effects of farm, parity, stage of lactation at the start of treatment, and season and bST treatment on variables of interest. The model was a split plot in time model, with all main effects and their interactions in the main plot tested by cow within farm x parity x stage of lactation x season x bST treatment interaction, and week and week x other main effects and interactions in the subplot, tested by the overall residual (Table 5-1). On farm 2, data were only collected on cows started in summer, and a new variable, group, was defined for data set A. Group 1 included data from cows on farm 1 started in summer, Group 2 included cows

started in fall, Group 3 included cows from farm 2, which were started in summer only.

Analysis by Peak Evaluation Algorithm

An inflammation event (IE) was defined as a $\log_2$(SCC) increase of at least one $\log_2$ unit from the preceding data point, when its value exceeded the baseline value characteristic for that cow at that time point by a value G, where G = SD[$\log_2$(SCC)] x g. The term SD[$\log_2$(SCC)] represents the within-assay standard deviation, g is the scaled residual.

The term SD[$\log_2$(SCC)] was calculated for the assay (measured by fluoro-opto-electronic procedure, Fossomatic^R) at each $\log_2$(SCC) level by a second order regression equation, calculated from reported values (Schmidt-Madsen, 1975): SD[$\log_2$(SCC)] = 0.4485 - 0.0692 x $\log_2$(SCC) + 0.0029 x [$\log_2$(SCC)]^2. In the $\log_2$(SCC) range of interest, SD[$\log_2$(SCC)] was decreasing with increasing $\log_2$(SCC). The threshold values for g to determine an IE were set such that duration and amplitude of the increase could be considered. An IE of one-week duration was detected when $\log_2$(SCC) exceeded the baseline by at least G = 11 x SD[$\log_2$(SCC)], i.e. the observed $\log_2$(SCC) exceeded the baseline by at least 11 standard deviations at that $\log_2$(SCC) level. An IE of two weeks duration was detected if $\log_2$(SCC) exceeded the baseline by G = 8 x SD[$\log_2$(SCC)] for at least two consecutive weeks, an IE of three weeks or longer was detected if $\log_2$(SCC) exceeded the baseline by G = 6 x SD[$\log_2$(SCC)] for at least

three consecutive weeks. It is to be noted that thresholds for g were set empirically. Sensitivity and specificity of IE detection by peak evaluation algorithm could be determined by comparison with visual evaluation. Altering thresholds for g will alter sensitivity and specificity of the detection method.

The baseline was calculated with the PULSAR program (Merriam and Wachter, 1982), which uses an algorithm for robust locally weighted regression and smoothing of scatterplots (Cleveland, 1979). The baseline represents long term trends of the SCC of individual cows. These trends include increases of SCC with increasing lactation number and stage of lactation (Brolund, 1985). The entire observation period for each cow was used as smoothing window: the smoothing window was set at 24 weeks in data sets A and B, and at 14 weeks in data set C. An option in PULSAR allows to determine if two consecutive peaks are considered as one or as two inflammation events. For these data sets, peaks were considered as split if the dip in $\log_2$(SCC) was larger than 10 x SD[$\log_2$(SCC)].

Three adjustments were made to the PULSAR output. First the baseline maximum accepted was set at $\log_2$(SCC) = 8.00 (SCC = 256,000). If the baseline calculated by PULSAR exceeded that value, calculation of g to determine presence of an IE was done manually with G calculated as difference of observed value minus baseline value of $\log_2$(SCC) = 8.00. Second,

$\log_2$(SCC) increases, characterized by PULSAR as peaks, in which $\log_2$(SCC) < 7.00 (SCC < 128,000 cells/ml), were not considered as inflammation events. Third, inflammation events were only considered if the increase of $\log_2$(SCC) to the previous value exceeded or was equal to 1, which represented a doubling of the SCC.

Variables obtained after analysis with PULSAR and subsequent adjustments included the number of inflammation events per cow, the duration, which allowed to calculate crude incidence rates of inflammation events (CIR) in IE x cow^{-1} x $year^{-1}$: CIR = [(number of IE / number of weeks at risk) x 52.14]. Number of weeks at risk were computed as number of weeks observed minus number of weeks inflamed. Data were stratified according to farm, season, treatment, parity, and stage of lactation group. The directly pooled point estimation of the effects of bST (data sets A and B) and season (data set C) for the incidence rate difference (IRD), the incidence rate ratio (IRR), confidence intervals for DIR and IRR, and an approximate test of hypothesis were calculated (Rothman, 1986). Other variables obtained through PULSAR for each cow were mean of the baseline values and direction of the baseline, the maximum amplitude of the residual G of an IE, and duration of inflammation events. Effects of treatment on these variables were evaluated by least squares analysis of variance, using models similar to those described previously.

Pearson coefficients of correlation were calculated for data from set A between average milk yield, $\log_2$(SCC), baseline value, maximum amplitude, duration of inflammation events and the individual cow incidence rate.

Results

Least Squares Analysis of Variance

Results from least squares means analysis of variance are in table 5-1. No effects of bST-treatment or season on $\log^2$(SCC) were detected, while parity had significant effects on $\log_2$(SCC) in all three data sets (for data set A, $\log_2$(SCC) for parity 1 was 5.85 ± 0.13, for parity 2+ it was 6.61 ± 0.10, $P < 0.01$).

Analysis by Peak Evaluation Algorithm

Figures 5-1, 5-2, 5-3 represent time series of $\log_2$(SCC) from three cows. Three baselines were calculated for each cow by PULSAR, using different parameters. Baseline 1 represents the baseline calculated with the width of the smoothing window set at 24 weeks. Thresholds for g to indicate the presence of an inflammation event were set to g = 11, 8, and 6, for inflammation events lasting 1 week, 2 weeks, or 3 or more weeks respectively. For baseline 2, width of smoothing window was reduced to 10 weeks, with the same g thresholds values as for baseline 1. For baseline 3, thresholds for g were set to

Table 5-1. Analysis of variance for $\log_2$(SCC) in data sets A, B, and C.

	Data Set					
	A		B		C	
SOURCE[1]	df	F-value	df	F-value	df	F-value
Farm (F)			1	0.87		
Season (S)					1	0.19
Group (G)	2	2.71+				
Parity (P)	1	9.69**	1	6.67*	1	6.94**
Stage of Lactation (LS)	2	0.47	2	0.87	2	1.26
Treatment (T)	1	1.63	1	0.76	1	1.61
P x SL			2	2.65+		
G x P x SL	2	2.72+				
P x T	1	11.10**	1	7.39**		
G x P x T	2	4.05*				
G x SL x T	4	2.18+				
P x SL x T	2	2.70+			2	3.18*
C(GxPxSLxT)[2]	139	19.07**				
C(FxPxSLxT)[3]			87	21.67**		
C(SxPxSLxT)[4]					105	14.61**
Week (W)	24	7.18**	23	7.05**	12	7.07**
W x F			13	4.02**		
W x S					8	2.71**
W x G	24	3.74**				
W x P	24	6.31**	23	4.59**	12	5.65**
W x SL	48	1.12	46	1.33+	24	1.02
W x T	24	0.73	23	0.69	12	0.72
MODEL	314	15.16	270	12.75	272	9.17
Residual	2405		1627		1106	

+: $P < 0.10$; *: $P < 0.05$; **: $P < 0.01$.
1) Non-significant interactions are not presented.
2) Error term for the effects above (main plot); MSE = 19.06.
3) Error term for the effects above; MSE = 22.98.
4) Error term for the effects above; MSE = 12.19.
5) R^2 for data set A: 0.66; for B: 0.68; for C: 0.69.

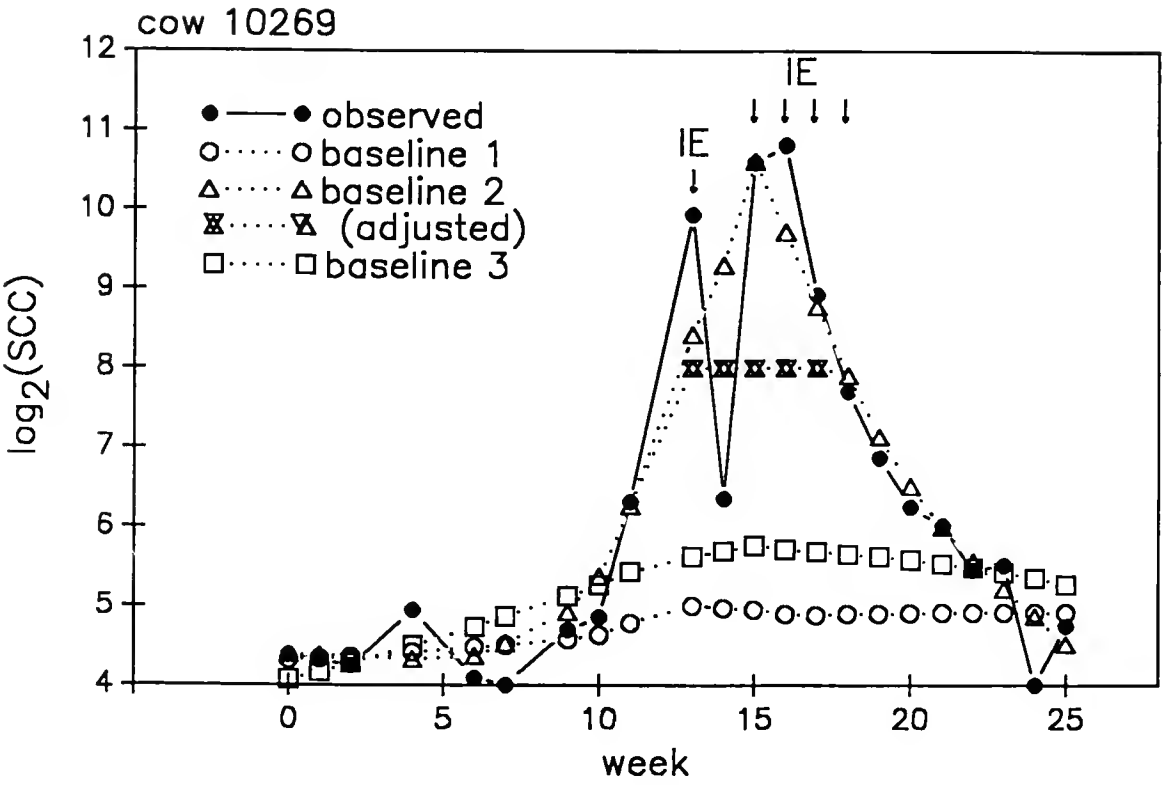

Figure 5-1. Observed $\log_2$(SCC) and baselines calculated by PULSAR with three sets of parameters. For baseline 1, the smoothing window was 24 weeks, and g threshold values were set at 11, 8, and 6 for inflammation events of 1, 2, or 3 or more weeks duration. With this baseline, 2 inflammation events were detected: one at week 13 (g_{max}=104.5), and one during weeks 15 to 18(g_{max}=150.2). Baseline 2 was calculated like baseline 1, except that smoothing window was 10 weeks. An inflammation event was detected at week 13 (g_{max}=32.2), and another at week 16 (g_{max}=28.0). When baseline 2 was adjusted so that it did not exceed $\log_2$(SCC)=8.0, g_{max} for the first inflammation event was 38.8, and the second inflammation event lasted for weeks 15 to 17 (g_{max}=70.2). Baseline 3 was calculated like baseline 1, except that g threshold values were set at 20, 15, and 12 for inflammation events of 1, 2, 3 or more weeks duration. Inflammation events were detected as for baseline 1 (g_{max} were 91.3 and 129.3). Baseline mean $\log_2$(SCC) were 4.7, 6.2, 5.2 for baselines 1, 2, and 3, and 5.9 for the adjusted baseline.

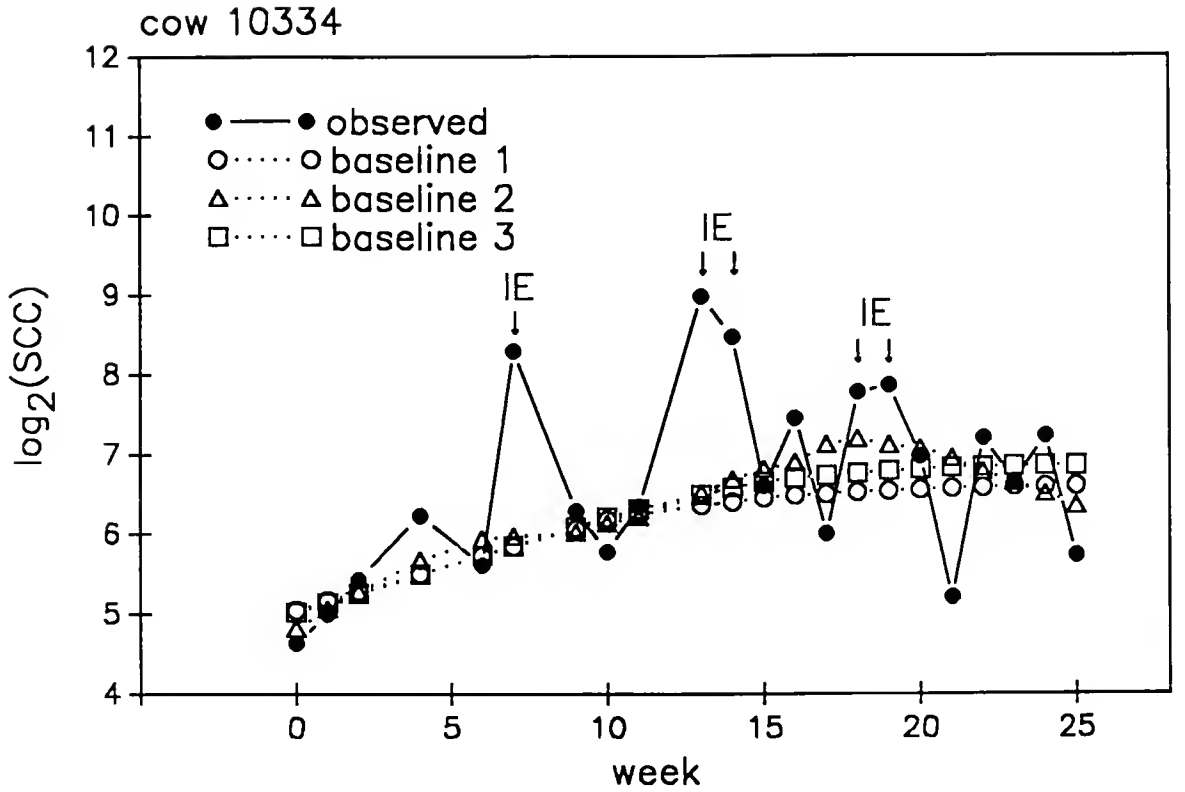

Figure 5-2. Observed $\log_2$(SCC) and baselines calculated by PULSAR with three sets of parameters (see legend of Figure 5-1). For baseline 1, inflammation events were detected at week 8 (g_{max}=33.1), weeks 14 to 15 (g_{max}=43.0), and weeks 19 to 20 (g_{max}=15.8). For baseline 2, where smoothing window width was reduced to 10 weeks, inflammation events were detected at week 8 (g_{max}=31.3), and weeks 14 to 15 (g_{max}=40.6). g value for week 20 was 8.9 . When g threshold values were increased (baseline 3), inflammation events were detected at week 8 (g_{max}=32.9), and weeks 14-15 (g_{max}=40.8), as for baseline 2. g value for week 20 was 12.8 . Baseline mean $\log_2$(SCC) were 6.1, 6.0, 6.2 for baselines 1, 2, and 3.

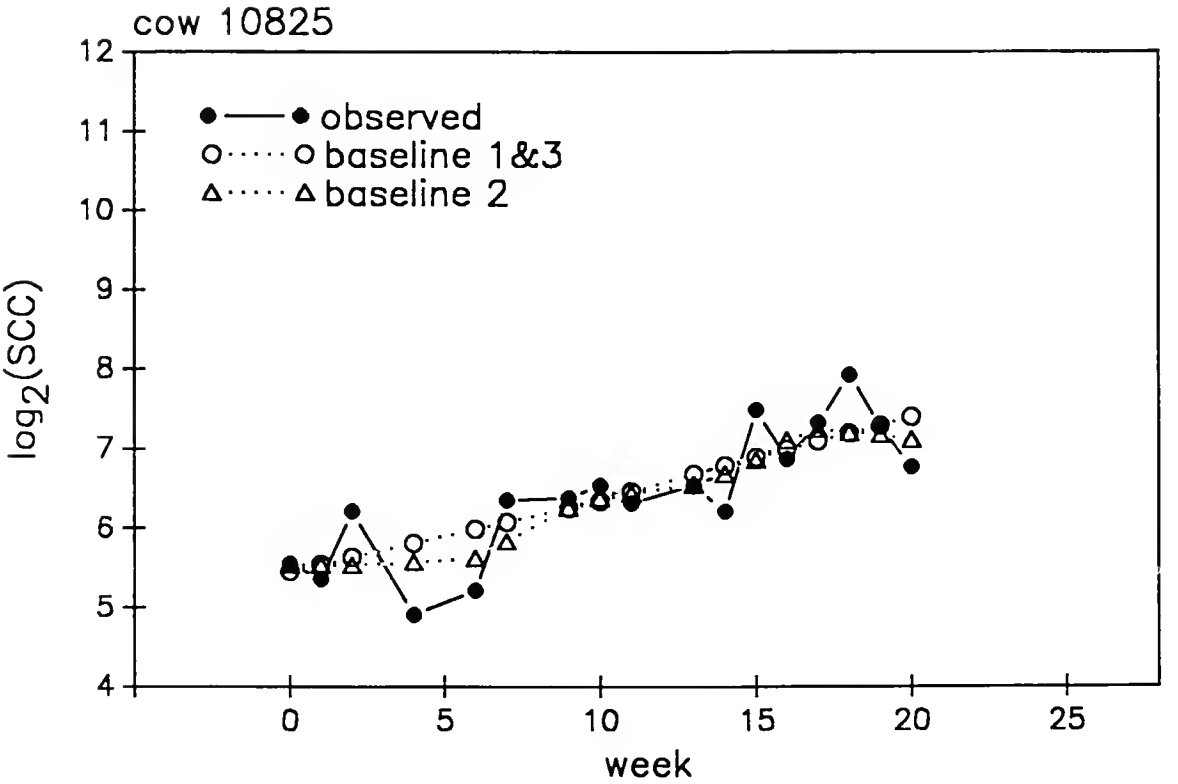

Figure 5-3. Observed log_2(SCC) and baselines calculated by PULSAR with three sets of parameters (see legend of Figure 5-1). No inflammation events were detected, using any of the three baselines. Baseline mean log_2(SCC) were 6.5, 6.4, 6.5 for baselines 1, 2, and 3.

g = 20, 15, and 12, with a smoothing window of 24 weeks. Adjustments specified in Materials and Methods to output of PULSAR were carried out for data from cow 10269 (Figure 5-1), where baseline 2 exceeded $\log_2(SCC)=8.00$ for weeks 13 to 17: for these two weeks baseline was maintained at $\log_2(SCC)=8.00$.

Mean $\log_2(SCC)$ was 6.18, 6.68, 6.47 (SEM=0.23) for cows 10269, 10334, 10825, and 11563 respectively. The incidence rate of inflammation events varied depending on which baseline was chosen. For cow 10269 (Figure 5-2), two inflammation events lasted a total of 5, 2, 4, and 5 weeks for baselines 1, 2, 2 adjusted, and 3. Thus the number of weeks at risk to suffer another inflammation event for cow 10269 was 16(=21-5), 19, 17, or 16 weeks, and the contribution to the CIR_{IE} of the cow population by cow 10269 was 6.5(= 5 / 16 x 52.14), 5.5, 6.1, and 6.5 x cow^{-1} x $year^{-1}$ for baselines 1, 2, 2 adjusted, and 3. For cow 10334, the contribution to the CIR_{IE} was 9.8, 5.8, and 5.8 for baselines 1, 2, and 3. The inflammation event at weeks 19-20 was only detected for baseline 1. For baseline 2 and 3, g was not sufficient (baseline 2: g_{19} = 6.8 and g_{20} = 8.9, g threshold for detection of a 2 week inflammation event was 8.0 for each of the two weeks; baseline 3: g_{19} = 11.7, g_{20} = 12.8, g threshold was 15.0). Maximum amplitude varied depending on baseline (Fig. 5-1, 5-2, 5-3). No inflammation events were detected for cow 10825.

Effects of bST and season on incidence rates of inflammation events

Data set A included 2,719 weekly observations for evaluation by the peak evaluation logarithm. One hundred and eight cows experienced at least one inflammation event for a total of 203 inflammation events. The directly pooled point estimate of the IRD of 0.96, with a lower limit of the 90 % confidence interval of 0.07 x cow^{-1} x $year^{-1}$, and the directly pooled point estimate of the IRR of 1.18, with a 90 % confidence interval of 0.91 to 1.52 indicated only a small association of bST treatment with increased IR_{IE}. The CHI^2 test for $IR_{treated}$ being different from $IR_{expected}$ was not significant (Table 5-2).

Data set B, using only cows started in summer, included 1,897 weekly observations for evaluation by PULSAR. One hundred and sixty-one inflammation events were detected in 79 of the 107 cows in the data set. The directly pooled point estimate of the IRD and the directly pooled point estimate of the IRR were lower than for data set A. The CHI^2test for $IR_{treated}$ being different from $IR_{expected}$ was not significant (Table 5-2).

Eighty-three inflammation events were detected in 1,378 observations of data set C. Fifty five of the 129 cows experienced at least one inflammation event. Stratification

Table 5-2. Effects of bST-treatment (data sets A and B) and summer season (data set C) on incidence rates of inflammation events, incidence rate differences and ratios, compared to effects of no-bST treatment or fall season.

Variable	DATA SET		
	A	B	C
Crude Total Incidence Rate [cow^{-1} x $year^{-1}$]	4.55	5.34	3.47
Crude Incidence Rate - no bST	4.22	5.07	
Crude Incidence Rate - bST [cow^{-1} x $year^{-1}$]	4.90	5.63	
Crude Incidence Rate - summer			4.31
Crude Incidence Rate - fall [cow^{-1} x $year^{-1}$]			2.91
Crude Incidence Rate Difference [cow^{-1} x $year^{-1}$]	0.68[a]	0.56[a]	1.40[b]
Incidence Rate Difference[+]	0.96[a]	0.88[a]	1.82[b]
(90 % confidence interval) [cow^{-1} x $year^{-1}$]	(0.07 1.85)	(-0.28 2.04)	(0.42 3.22)
Crude Incidence Rate Ratio	1.16[c]	1.11[c]	1.48[d]
Incidence Rate Ratio[+]	1.18[c]	1.11[c]	1.57[d]
(90 % confidence interval)	(0.91 1.52)	(0.84 1.46)	(1.07 2.31)
CHI^2	1.23	0.47	4.40*

+: directly pooled point estimate
*: $P < 0.05$

a: (bST - no bST)
b: (summer - fall)
c: (bST / no bST)
d: (summer / fall)

of data included strata for bST treatment groups. The IRD (summer minus fall) of 1.82 x cow^{-1} x $year^{-1}$ with a 90% confidence interval from 0.42 to 3.22 x cow^{-1} x $year^{-1}$, and the IRR (summer / fall) of 1.57 with a 90% confidence interval from 1.07 to 2.31 indicated an association between summer season and increased incidence rate of inflammation events. The CHI^2 test for IR_{summer} being different from $IR_{expected}$ was significant ($P < 0.05$; Table 5-2). The discrepancies between the crude estimates and the directly pooled point estimates, which are unconfounded, indicate that the crude results were biased by confounding factors.

Effects of bST and season on characteristics of inflammation events

The duration of IE ranged between 1 and 8 weeks for events completed within the experimental period, while one cow had elevated SCC for 13 weeks up to the end of the experimental period. The longest IE in cows started in fall lasted 4 weeks. Data for baseline, maximum amplitude, and duration of IE were analyzed by least squares analysis of variance. In A (Table 5-3), baselines tended to be lower in fall (Group 2) and were higher for cows in second and higher parity. Maximum amplitude decreased with increasing lactation stage. Duration of inflammation events in a cow was higher for bST treated cows than for control cows. For data set B (Table 5-4), baselines were higher for second and higher parity cows. Baselines increased on farm 1 with increasing

Table 5-3. Least squares means ± SEM of observed SCC, baseline SCC, maximum amplitude and duration of inflammation events for data set A.

Variable	Observed SCC	Baseline SCC	Maximum Amplitude	Duration of Inflammation Events
	----------[$\log_2$(SCC)]----------			[weeks]
Group 1	6.6 ± 0.1**	6.2 ± 0.1+	2.2 ± 0.1+	1.6 ± 0.2**
Group 2	5.9 ± 0.1	5.7 ± 0.1	2.5 ± 0.2	1.2 ± 0.3
Group 3	6.5 ± 0.2	6.0 ± 0.1	2.7 ± 0.2	2.6 ± 0.2
Parity 1	5.9 ± 0.1[a]	5.6 ± 0.1[a]	2.5 ± 0.2	1.7 ± 0.3
Parity 2+	6.8 ± 0.1[b]	6.3 ± 0.1[b]	2.5 ± 0.1	1.9 ± 0.1
Stage of Lactation				
1	6.4 ± 0.1	5.9 ± 0.1+	2.8 ± 0.2*	1.8 ± 0.2
2	6.1 ± 0.1	5.8 ± 0.1	2.5 ± 0.2	1.7 ± 0.2
3	6.5 ± 0.1	6.2 ± 0.1	2.1 ± 0.2	1.8 ± 0.2
bST Control	6.2 ± 0.1	5.8 ± 0.1	2.7 ± 0.1	1.5 ± 0.2[a]
bST Treatment	6.5 ± 0.1	6.0 ± 0.1	2.7 ± 0.1	2.1 ± 0.2[b]

Least squares means with different superscripts for a variable are significantly different: ab: $P < 0.01$.

\+ Effect of group and stage of lactation: $P < 0.10$.
* Effect of stage of lactation: $P < 0.05$.
** Effect of group: $P < 0.01$.

Table 5-4. Least squares means ± SEM of observed SCC, baseline SCC, maximum amplitude and duration of inflammation events for data set B.

Variable	Observed SCC	Baseline SCC	Maximum Amplitude	Duration of Inflammation Events
	----------	[$\log_2$(SCC)]	----------	[weeks]
Farm 1	6.2 ± 0.1	5.9 ± 0.1	2.2 ± 0.1[a]	1.5 ± 0.2[e]
Farm 2	6.2 ± 0.1	5.7 ± 0.3	3.1 ± 0.2[b]	2.4 ± 0.2[f]
Parity 1	5.6 ± 0.4[c]	5.3 ± 0.3[c]	2.7 ± 0.2	1.9 ± 0.3
Parity 2+	6.8 ± 0.1[d]	6.2 ± 0.1[d]	2.6 ± 0.1	2.1 ± 0.2
Stage of Lactation				
1	6.4 ± 0.2	5.8 ± 0.2	2.9 ± 0.2	2.1 ± 0.3
2	5.9 ± 0.3	5.5 ± 0.3	2.8 ± 0.2	1.9 ± 0.3
3	6.4 ± 0.3	6.0 ± 0.3	2.3 ± 0.2	2.0 ± 0.3
bST Control	5.9 ± 0.3	5.5 ± 0.2	2.8 ± 0.2	1.6 ± 0.2[e]
bST Treatment	6.5 ± 0.3	6.1 ± 0.3	2.6 ± 0.2	2.4 ± 0.2[f]

Least squares means with different superscripts for a variable are significantly different: ab: $P < 0.10$; cd: $P < 0.05$; ef: $P < 0.01$.

+ Effect of lactation stage: $P < 0.10$.

Table 5-5. Least squares means ± SEM of observed SCC, baseline SCC, maximum amplitude and duration of inflammation events for data set C.

Variable	Observed SCC	Baseline SCC	Maximum Amplitude	Duration of Inflammation Events
	----------[$\log_2$(SCC)]----------			[weeks]
Summer	5.9 ± 0.1	5.6 ± 0.1	2.3 ± 0.2	1.4 ± 0.2
Fall	5.8 ± 0.1	5.5 ± 0.1	2.4 ± 0.2	1.5 ± 0.2
Parity 1	5.6 ± 0.2[e]	5.4 ± 0.1[c]	2.3 ± 0.2	1.3 ± 0.2
Parity 2+	6.1 ± 0.1[f]	5.8 ± 0.1[d]	2.5 ± 0.2	1.6 ± 0.2
Stage of Lactation				
1	5.6 ± 0.2	5.3 ± 0.2+	2.7 ± 0.2+	1.3 ± 0.2
2	5.8 ± 0.2	5.6 ± 0.2	2.4 ± 0.2	1.5 ± 0.2
3	6.0 ± 0.2	5.8 ± 0.2	2.0 ± 0.2	1.5 ± 0.2
bST Control	5.7 ± 0.1	5.5 ± 0.1	2.6 ± 0.2[a]	1.4 ± 0.2
bST Treatment	6.0 ± 0.1	5.7 ± 0.1	2.2 ± 0.2[b]	1.5 ± 0.2

Least squares means with different superscripts for a variable are significantly different: ab: $P < 0.10$; cd: $P < 0.05$; ef: $P < 0.01$.

+ Effect of lactation stage: $P < 0.10$.

lactation stage, while on farm 2, baselines in stage of lactation group 2 were lower than in group 1 and 3 (data not shown). As for data set A, duration of inflammation events was higher for bST-treated cows. For data set C (Table 5-5), baselines were again higher for second and higher parity cows, and tended to increase with lactation stage. Maximum amplitude tended to decrease with increasing lactation stage. No seasonal effects on variables in table 5-5 were detected.

In data set A, the baseline increased in 63.8 % of the cows by at least 0.5 units of $\log_2$(SCC) over the duration of the experiment, which corresponds to the expectation of increasing SCC with advancing stage of lactation. Baselines decreased in 9.9 % of the cows, mainly in cows with low SCC.

Correlation coefficients are displayed in table 5-6. The coefficient for single observation pairs (n=2623) for correlation of milk yield and $\log_2$(SCC) in A was -0.28. Correlation coefficients of milk yield with other variables tested were low, although significant. Thus, increase in duration of inflammation events for a cow and increase in $\log_2$(SCC) were associated with a decrease in milk yield at a similar level. Concurrently $\log_2$(SCC) and duration of inflammation events were correlated. No correlations between $\log_2$(SCC), milk yield, and maximum amplitude were detected.

Table 5-6. Pearson Correlation Coefficients for individual cow means, derived from data set A.

	$\log_2$(SCC)	Baseline	Maximum Amplitude	Duration	IR
Milk Yield	-0.27**	-0.26**	0.01	-0.33**	-0.17*
$\log_2$(SCC)	1.00	0.95**	0.17	0.40**	0.68**

*: $P < 0.05$; **: $P < 0.01$.

Discussion

The contamination of the mammary gland with a pathogen, with possibly resulting infection and inflammation is a sporadic event, and of varying duration. Conventional analysis, using least squares analysis of variance, does not completely describe the dynamic nature of SCC data. Given the variable behavior of pathogens, and the variable SCC response, it was useful to develop a system to evaluate inflammation events, based on SCC data, while accounting for variation due to assay, diurnal variation, and long term trends. Variable thresholds for determining mastitis in cows have been proposed: Dohoo et al. (1981), and Sheldrake et al. (1983) proposed age-related or pathogen-related thresholds for classifying milk as mastitic. The problem with this approach is that SCC do not necessarily return to pre-infection levels, and therefore thresholds have to be kept high to avoid false-positive classifications. Mattila (1985) proposed the use of inter-quarter ratios for N-acetyl-β-D-glucosaminidase in milk, which enables comparison of inflamed to noninflamed control quarters in the same cow. This method requires many samples, and poses a problem in cows with continuous alterations, for example due to previous infections and inflammations (Linzell et al., 1974), or in cows with more than one quarter inflamed.

Use of the peak evaluation method proposed here would not have these difficulties, because a dynamic threshold is set,

which depends on the individual cow, her age and lactation stage, and her udder health history. Besides detecting an incident inflammation event, the evaluation of the severity of the inflammation is possible, by comparing the observed SCC to a calculated normal level, i.e. to the baseline, and assessing duration and amplitude of SCC changes.

It is to be noted that parameter values in the input to PULSAR are set empirically. By altering parameters, sensitivity and specificity for diagnosis of an inflammation event are altered; sensitivity and specificity also depend on the prevalence of inflammation events in a population (McDermott et al., 1982). It is the responsibility of the investigator to set the thresholds at which an inflammation event will be detected by PULSAR. Parameters such as the width of the smoothing window, or the threshold g values, when changed, can alter the types of SCC increases reported as inflammation events. Once parameters, and conditions for eventual manual adjustments are set for a population, then the use of PULSAR will guarantee rigor in the evaluation of SCC series from all cows in the investigated population. Such rigor cannot be guaranteed for visual procedures.

The power of this approach using the peak evaluation procedure followed by the directly pooled point estimation of a uniform effect (Rothman, 1986) could be demonstrated in comparison to results by least squares analysis of variance. Although no seasonal effects could be detected by analysis of

variance of data set C, an elevated incidence rate of inflammation events could be detected in summer. Average baseline values, maximum amplitude and duration of inflammation events provided additional information on the resistance and health status of the mammary gland. These variables were not different for summer and fall. Thus it appears that the elevated incidence of inflammation events was due to increases in challenge to the mammary gland during the hotter and more humid summer months. No effects of bST treatment on incidence rates could be detected, but duration of inflammation events was higher in bST treated cows in A and B. This could be an indication for a more protracted response of bST treated cows to a bacterial challenge, i.e. the possibility exists that bST cows do not eliminate infections as efficiently as non-treated cows.

Baselines were higher for cows in second and higher parity, an expected result, since SCC increases with increasing parity (Ali and Shook, 1983). Effects of stage of lactation were not as clear, although a tendency existed for higher baselines to occur later in lactation in data set C. Also amplitude of inflammation events decreased with increasing lactation stage, which could indicate a lower efficiency at eliminating infections with advancing stage of lactation.

In summary, the peak evaluation algorithm applied to SCC time series is a useful procedure for characterizing

inflammation events in the mammary gland. The method is based on determining a dynamic threshold for individual cows to detect inflammation in the udder. It also allows the investigator to consider other measures of udder health, like amplitude and duration of inflammation events. By applying this method, seasonal and bST-related effects on SCC could be detected.

Further validation studies should determine if incidence rates of inflammation events can be associated to incidence rates of contamination and of infection. If a high degree of association exists, then analysis of SCC time series by a peak evaluation algorithm and subsequent analysis of incidence rates of inflammation events will provide a relatively cheap means of determining infectious events in herd studies.

CHAPTER 6
GENERAL DISCUSSION

Animal health is an important factor for production efficiency of food animals. The immune system, which is responsible for protection and recovery of an animal from infectious diseases, has long been recognized as being influenced by the environment. Identification of environmental factors which affect the activity of the immune system could lead to management procedures to enhance immune function. In the present study, it was shown that heat stress and supplemental bovine somatotropin affect components of the immune system.

Several reports have indicated that incidence of mastitis increased during summer (Roman-Ponce et al., 1977; Morse et al., 1988). These findings were further strengthened by the analysis in chapter 5, which showed that during summer, incidence rates of inflammation events in the mammary glands of lactating cows increased, although duration and severity were not affected. It is not clear whether increases in disease incidence result from increased challenge by microorganisms, or if increases are due to compromised immune function. Results from these studies suggest that elevated temperatures can have severe negative effects on function of

polymorphonuclear leukocytes and lymphocytes in vitro, but that many of these effects are not seen when heat stress is applied in vivo. Nevertheless some in vivo effects may be important.

Heat stress in vitro inhibited migration, oxidative metabolism and phagocytosis by polymorphonuclear leukocytes, while in vivo heat stress reduced chemotactic migration in vitro (Chapter 2). Concurrently, in vivo heat stress inhibited migration of leukocytes into the mammary gland after intra-mammary oyster glycogen infusion (Chapter 4). It is of interest that basal somatic cell counts in cows exposed to heat stress were higher than when cows were in thermoregulated environment. In heat-stressed cows, intra-cisternal temperatures are closer to homeothermic temperatures, since intra-cisternal temperatures are lower than body core temperatures (Chapter 2). The small, but significant increase of somatic cell counts thus could be due to enhanced migration into the mammary gland. In this case, increases of somatic cell counts cannot necessarily be associated with infection or disease.

No effects of heat stress on oxidative metabolism and phagocytosis by polymorphonuclear leukocytes were measured when heat stress was applied in vivo. It is likely that compensating mechanisms, present in the organism to protect cell function in hyperthermic animals, are not available to cells in the in vitro system.

In vitro heat stress severely reduced proliferation of lymphocytes after mitogen stimulation, while in vivo heat stress had no inhibiting effect on lymphocytes. Conversely, cells obtained from heat-stressed cows were less affected by elevated incubation temperatures than cells from cows in thermoregulated environment. The hypothesis that this could be due to alterations in cell populations and sub populations, as had been shown for hyperthermic humans and rats (Downing et al., 1988), was not verified, since no differences in percentages of $CD2^+$, $CD4^+$, $CD8^+$-cells, or B lymphocytes could be detected. Another protective mechanism for cells from heat-stressed cattle could be the synthesis of heat shock proteins during in vivo heat stress, which could confer a certain degree of thermotolerance to cells in a subsequent in vitro heat-stress environment, as was reported by Mizzen and Welch (1988).

Recombinantly derived bovine somatotropin is likely to play an important role in dairy production, and it is of importance to quantify its effects on immune function and disease resistance. The working hypothesis was that bST would enhance immune function (Edwards et al., 1988; Heyneman et al., 1989), in particular when compromised (Chatterton et al., 1973; Davila et al., 1987). No effects of supplemental bST on polymorphonuclear leukocytes were measured, but duration of inflammation events, assessed by increases of somatic cell counts (Chapter 5), was longer in bST treated cows.

Somatotropin at pharmacological doses was shown to be mitogenic to lymphocytes and it also conferred a certain degree of thermotolerance to mitogen stimulated lymphocytes after in vitro and in vivo heat exposure. In vivo treatment with bST did not alter lymphocyte populations and subpopulations, and it is possible that bST stimulation increased synthesis of heat shock proteins, as had been shown for other mitogens (Haire et al., 1988), which protect cells from heat insults (Mizzen an Welch, 1988). Thus it is possible that bST improves function of the immune system, especially in heat-stressed cows. Nevertheless, caution has to be exerted, since bST supplementation makes cows more susceptible to heat stress (Chapter 4). Somatotropin treated cows had higher rectal temperatures than cows treated with placebo, and symptoms of heat strain were much more severe than symptoms in cows treated with placebo. Nevertheless, bST treated cows produced more milk, even when heat stressed, and management procedures have to be adapted to protect bST treated cows from severe heat-stress conditions.

To conclude, heat stress depressed certain components of the immune system. Supplementation with bST did not affect function of polymorphonuclear leukocytes, while it did improve lymphocyte function. Thus, if cows are well managed to reduce effects of summer heat stress in sub-tropical climates, it is possible that bST supplementation enhances the immune system and animal health.

REFERENCE LIST

Abe, H., M.E. Molitch, J.J. Van Wyk, and L.E. Underwood. 1983. Human growth hormone and somatomedin C suppress the spontaneous release of growth hormone in unanesthetized rats. Endocrinology 113:1319.

Ali, A.K.A., and G.E. Shook. 1980. An optimum transformation for somatic cell concentration in milk. J. Dairy Sci. 63:487.

Anderson, D.J., and J.A. Hill. 1988. Cell-mediated immunity in infertility. Am. J. Reprod. Immunol. Microbiol. 17:22.

Arrenbrecht, S. 1974. Specific binding of growth hormone to thymocytes. Nature 252:255.

Arthur, J.R., R. Boyne, H.A.O. Hill, and M.J. Okolow-Zubkowska. 1981. The production of oxygen-derived radicals by neutrophils from selenium-deficient cattle. FEBS Lett. 135:1981.

Asakawa, K., J.A. Hedo, A. McElduff, D.G. Rouiller, M.J. Waters, and P. Gorden. 1986. The human growth hormone receptor of cultured human lymphocytes. Biochem. J. 238:379.

Asherson, G.L., V. Colizzi, and M. Zambala. 1986. An overview of T-suppressor cell circuits. Ann. Rev. Immunol. 4:37.

Bachman, K.C., F. Elvinger, H.H. Head. 1990. La Somatotropine: Montée laiteuse et production laitière. In Conceptions modernes de la lactation. J. Martinet and L.M. Houdebine, ed. Institut National de Recherche Agronomique, Jouy-en-Josas, France.

Badwey, J.A. and M.L. Karnovsky. 1980. Active oxygen species and the functions of phagocytic leukocytes. Ann. Rev. Biochem. 49:695.

Bakken, G. 1981. Subclinical mastitis in Norwegian dairy cows. Prevalence rates and epidemiological assessments. Acta Agric. Scand. 31:273.

Baldwin, C.L., A.J. Teale, J. Naessens, B.M. Goddeeris, W.D. MacHugh, and W.I. Morrison. 1986. Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: Similarity to lymphocytes defined by human T4 and murine L3T4. J. Immunol. 136:4385.

Bauman, D.E., P.J. Eppard, M.J. DeGeeter, and G.M. Lanza. 1985. Response of high producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. J. Dairy Sci. 68:1352.

Bauman, D.E., D.L. Hard, B.A. Crooker, M.S. Partridge, K. Garrick, L.D. Sandles, H.N. Erb, S.E. Franson, G.F. Hartnell, and R.L. Hintz. 1989. Long-term evaluation of a prolonged release formulation of N-methionyl bovine somatotropin in lactating dairy cows. J. Dairy Sci. 72:642.

Berczi, I. 1986. The effects of growth hormone and related hormones on the immune system. Page 133 in Pituitary Function and Immunity. I. Berczi, ed. CRC Press, Boca Raton, FL.

Berning, L.M., M.J. Paape, R.H. Miller, and R.A. LeDane. 1987. N-acetyl-β-D-glucosaminidase activities, milk somatic cell counts, and blood leukocyte and erythrocyte counts in cows after heat-induced stress or after intravenous administration of adrenocorticotropic hormone. Am. J. Vet. Res. 48:1157.

Berning, L.M., M.J. Paape, A.J. Guidry, R.R. Peters, and S.K. Salgar. 1989. Comparison of antibody-binding characteristics of bovine peripheral blood and mammary gland neutrophils (PMN). J. Dairy Sci. 72(Suppl.1):10.(Abstr.)

Blackburn, P.S. 1966. The variation in the cell count of cow's milk throughout lactation and from one lactation to the next. J. Dairy Res. 33:193.

Blatt, J., S. Wenger, S. Stitely, and P.A. Lee. 1987. Lack of mitogenic effects of growth hormone on human leukemic lymphoblasts. Eur. J. Pediatr. 146:257.

Bodoh, G.W., W.J. Battista, L.H. Schultz, and R.P. Johnston, Jr. 1976. Variation in somatic cell counts in dairy herd improvement milk samples. J. Dairy Sci. 59:1119.

Bodoh, G.W., R.E. Pearson, W.D. Schultz, and R.H. Miller. 1981. Variation in Wisconsin Mastitis Test scores of bucket milk samples and relationship to bacterial infections. J. Dairy Sci. 64:123.

Booth, J.M. 1985. Bulk milk somatic cell counting: Methods in use and a proposal for the standard presentation of data. Kieler Milchwirtschaftl. Forschungsber. 37:274.

Boyne, R., and J.R. Arthur. 1979. Alterations of neutrophil function in selenium-deficient cattle. J. Comp. Path. 89:151.

Brandon, M.R., D.L. Watson and A.K. Lascelles. 1971. The mechanism of transfer of immunoglobulin into mammary secretion of cows. Aust. J. Exp. Biol. Med. Sci. 49:613.

Brandt, A.E. 1938. Test of significance in reversal or switchback trials. Iowa Agr. Expt. Sta., Res. Bull. 234:60.

Bray, D.R., M.A. DeLorenzo, F.C. Elvinger, D.K. Beede, J.K. Shearer, P.A. Reed, J. Boosinger. 1989. Cooling ponds and milk quality. Page 188 in Proceedings, Natl. Mastitis Council, Tampa, FL.

Brazeau, P., W. Vale, R. Burgus, N. Ling, M. Butcher, J. River, and R. Guillemin. 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179:77.

Brock, J.H., E.D. Steel, and B. Reiter. 1975. The effect of intramuscular and intramammary vaccination of cows on antibody levels and resistance to intramammary infection by Staphylococcus aureus. Res. Vet. Sci. 19:152.

Brolund, L. 1985. Cell Counts in Bovine Milk. Causes of variation and applicability for diagnosis of subclinical mastitis. Acta. Vet. Scand. Suppl. 80:1.

Brooker, B.E. 1978. Characteristic cell fragments in bovine milk. J. Dairy Res. 45:21.

Brooks, B.W., D.A. Barnum, and A.H. Meek. 1982. A survey of mastitis in selected Ontario dairy herds. Can. J. Comp. Med. 48:146.

Bruecker, K.A., and L.W. Schwartz. 1982. Bovine peripheral blood polymorphonuclear neutrophil chemotactic response to Pasteurella haemolytica or zymosan-activated serum. Am. J. Vet. Res. 43:1879.

Buffington, D.E., A. Collazo-Arocho, G.H. Canton, D. Pitt, W.W. Thatcher, R.J. Collier. 1981. Black Globe-Humidity Index (BGHI) as comfort equation for dairy cows. Trans. ASAE 24:711.

Cambier, J.C., L.B. Justement, M.K. Newell, Z.Z. Chen, L.K. Harris, V.M. Sandoval, M.J. Klemsz, and J.T. Ransom. 1987. Transmembrane signals and intracellular "second messengers" in the regulation of quiescent B-lymphocyte activation. Immunol. Rev. 95:37.

Carroll, E.J., R. Mueller, L. Panico. 1982. Chemotactic factors for bovine leukocytes. Am. J. Vet. Res. 43:1661.

Chalupa, W., and D.T. Galligan. 1989. Nutritional implications of somatotropin for lactating cows. J. Dairy Sci. 72:2510.

Chandler, R.L., A.W.D. Lepper and J. Wilcox. 1969. Ultrastructural observations on the bovine teat duct. J. Comp. Path. 79:315.

Chatterton, R.T., Jr., C.L. Murray, and L. Hellman. 1973. Endocrine effects on leukocytopoiesis in the rat. I. Evidence for growth hormone secretion as the leukocytopoietic stimulus following acute cortisol-induced lymphopenia. Endocrinology 92:775.

Chew, B.P. 1987. Vitamin A and β-carotene on host defense. J. Dairy Sci. 70:2732.

Chilliard, Y. 1988. Rôles et mécanismes d'action de la somatotropine (hormone de croissance) chez le ruminant en lactation. Reprod. Nutr. Dévelop. 28:39.

Christison, G.I., and H.D. Johnson. 1972. Cortisol turnover in heat-stressed cows. J. Animal Sci. 33:1005.

Ciavarra, R.P., S. Silvester, and T. Brody. 1987. Analysis of T-cell subset proliferation at afebrile and febrile temperatures: Differential response of Lyt-$1^{+}23^{-}$ lymphocytes to hyperthermia following mitogen and antigen stimulation and its functional consequence of development of cytotoxic lymphocytes. Cell. Immunol. 107:293.

Cleveland, W.S. 1979. Robust locally weighted regression and smoothing scatterplots. J. Am. Stat. Assoc. 74:829.

Colditz, I.G., and D.L. Watson. 1982. Effect of immunisation on the early influx of neutrophils during staphylococcal mastitis in ewes. Res. Vet. Sci. 33:146.

Cole, W.J., P.J. Eppard, G.M. Lanza, R.L. Hintz, K.S. Madsen, S.E. Franson, T.C. White, W.E. Ribelin, B.G. Hammond, S.C. Bussen, R.K. Leak, and L.E. Metzger. 1988. Response of lactating dairy cows to multiple injections of sometribove, USAN (recombinant methionyl bovine somatotropin) in a prolonged release system. Part II. Health and reproduction. J. Dairy Sci. 71(Suppl.1):184.(Abstr.)

Collins, R.A, and G. Oldham. 1986. Proliferative responses and IL-2 production by mononuclear cells from bovine mammary secretions, and the effect of mammary secretions on peripheral blood lymphocytes. Immunology 58:647.

Concha, C, O. Holmberg, and B. Morein. 1978. Proportion of B- and T-lymphocytes in normal bovine milk. J. Dairy Res. 45:287.

Convey, E.M., L.S. Miller, and H.A. Tucker. 1971. Acute effects of 9α-fluoroprednisolone acetate (9α-FFA) on blood leukocytes and somatic cell content of milk. J. Dairy Sci. 54:360.

Craven, N. 1983. Generation of neutrophil chemoattractants by phagocytosing bovine mammary macrophages. Res. Vet. Sci. 35:310.

Craven, N. 1986. Chemotactic factors for bovine neutrophils in relation to mastitis. Comp. Immun. Microbiol. Infect. Dis. 9:29.

Craven, N., and M.R. Williams. 1985. Defences of the bovine mammary gland against infection and prospects for their enhancement. Vet. Immunol. Immunopathol. 10:71.

Davis, S.L. 1975. Somatostatin: its influence on plasma levels of growth hormone, prolactin and thyrotropin in sheep. J. Anim. Sci. 40:911.

Cullen, G.A. 1968. Cell counts throughout lactation. Vet. Rec. 83:125.

Curtis, S.E. 1983. Environmental management in animal agriculture. The Iowa State Press, Ames, IA.

Davila, D.R., S. Brief, J. Simon, R.E. Hammer, R. Brinster, and K.W. Kelley. 1987. Role of growth hormone in regulating T-dependent immune events in aged, nude, and transgenic rodents. J. Neurosci. Res. 18:108.

Davis, W.C., S. Marusic, H.A. Lewin, G.A. Splitter, L.E. Perryman, T.C. McGuire, and J.R. Gorham. 1987. The development of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. Vet. Immunol. Immunopathol. 15:337.

Dehoff, M.H., R.G. Elgin, R.J. Collier, and D.R. Clemmons. 1988. Both Type I and II insulin-like growth factor receptor binding increase during lactogenesis in bovine mammry tissue. Endocrinology 122:6.

Derbyshire, J.B. 1960. Experimental studies on the occurrence of staphylococcal antitoxin in the whey of cows. Res. Vet. Sci. 1:350.

Doane, R.M., S.P. Oliver, R.D. Walker, and E.P. Shull. 1987. Experimental infection of lactating bovine mammary glands with Streptococcus uberis in quarters colonized by Corynebacterium bovis. Am. J. Vet. Res. 48:749.

Dohoo, I.R., I. McMillan, and A.H. Meek. 1981a. The effects of storage and method of fixation on somatic cell counts in bovine milk. Can. J. Comp. Med. 45:335.

Dohoo, I.R., A.H. Meek, and S.W. Martin. 1984. Somatic cell counts in bovine milk: relationships to production and clinical episodes of mastitis. Can. J. Comp. Med. 48:130.

Dohoo, I.R., A.H. Meek, S.W. Martin, and D.A. Barnum. 1981b. Use of total and differential somatic cell counts from composite milk samples to detect mastitis in individual cows. Can. J. Comp. Med. 45:8.

Donaldson, A.I. 1978. Factors influencing the dispersal, survival and deposition of airborne pathogens of farm animals. Vet. Bull. 48:83.

Dorf, M.E., and B. Benacerraf. 1984. Suppressor cells and immunoregulation. Ann. Rev. Immunol. 1:127.

Downing, J.F., H. Martinez-Valdez, R.S. Elizondo, E.B. Walker, and M.W. Taylor. 1988. Hyperthermia in humans enhances interferon-γ synthesis and alters the peripheral lymphocyte population. J. Interferon Res. 8:143.

Downing, J.F., and M.W. Taylor. 1987. The effect of in vivo hyperthmia on selected lymphokines in man. Lymphokine Res. 6:103.

Duff G.W., and S.C. Durum. 1983. The pyrogenic and mitogenic actions of interleukin-1 are related. Nature 304:449.

Duhamel, G.E., D. Bernoco, W.C. Davis, and B.I. Osburn. 1987. Distribution of T and B. lymphocytes in mammary dry secretions, colostrum and blood of adult dairy cattle. Vet. Immunol. Immunopathol. 14:101.

Duitschaever, C.L. and G.C. Ashton. 1972. Variations of somatic cells and neutrophils in milk throughout lactation. J. Milk Food Technol. 35:197.

Dulin, A.M., M.J. Paape, and S.C. Nickerson. 1988. Comparison of phagocytosis and chemiluminescence by blood and mammary gland neutrophils from multiparous and nulliparous cattle. Am. J. Vet. Res. 49:172.

Eberhart, R.J., H.C. Gilmore, L.J. Hutchinson, and S.B. Spencer. 1979. Somatic cell counts in DHI samples. Page 32 in Proc. 18th Annu. Meeting, National Mastitis Council, Louisville, KY.

Edwards, C.K.,III, S.M. Ghiasuddin, J.M. Schepper, L.M. Yunger, and K.W. Kelley. 1988. A newly defined property of somatotropin: Priming of macrophages for production of superoxide anion. Science 239:769.

Ellis, J.A., C.L. Baldwin, W.D. MacHugh, A. Bensaid, A.J. Teale, B.M. Goddeeris, and W.I. Morrison. 1986. Characterization by a monoclonal antibody and functional analysis of a subset of bovine T lymphocytes that express BoT8, a molecule analogous to human CD8. Immunology 58:351.

Elvinger, F. 1983. Verbreitung von B-Streptokokken und Untersuchungen von Hygienefaktoren in Rinderzuchtbeständen im Kreis Winsen (Luhe). Dr. med. vet. diss., Tierärztliche Hochschule, Hannover, Germany.

Elvinger, F., H.H. Head, C.J. Wilcox, R.P. Natzke, and R.G. Eggert. 1988. Effects of administration of bovine somatotropin on milk yield and composition. J. Dairy Sci. 71:1515.

Emanuelson, U., and E. Persson. 1984. Studies on somatic cell counts in milk from Swedish dairy cows. I. Non-genetic causes of variation in monthly test-day results. Acta Agric. Scand. 34:33.

Engleman, E.G. C.J. Benike, F.C. Grumet, and R.L. Evans. 1981. Activation of human T lymphocyte subsets: Helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility and antigens. J. Immunol. 127:2124.

Eppard, P.J., D.E. Bauman, C.R. Curtis, H.N. Erb, G.M. Lanza, and M.J. DeGeeter. 1987. Effect of 188-day treatment with somatotropin on health and reproductive performance of lactating dairy cows. J. Dairy Sci. 70:582.

Eppard, P.J., D.E. Bauman, and S.N. McCutcheon. 1985. Effect of dose of bovine growth hormone on lactation of dairy cows. J. Dairy Sci. 68:1109.

Eppard, P.J., G.M. Lanza, S. Hudson, W.J. Cole, R.L. Hintz, T.C. White, W.E. Ribelin, B.G. Hammond, S.C. Bussen, R.K. Leak, and L.E. Metzger. 1988. Response of lactating dairy cows to multiple injections of sometribove, USAN (recombinant methionyl bovine somatotropin) in a prolonged release system. Part I. Production response. J. Dairy Sci. 71(Suppl.1):184.(Abstr.)

Fernandez, H.N., P.M. Henson, A. Otani, and T.E. Hugle. 1978. Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis in vitro and under simulated in vivo conditions. J. Immunol. 120:109.

Fernando, R.S., and S.L. Spahr. 1983. Effects of milking interval on selected milk constituents from normal and infected quarters. J. Dairy Sci. 66:1155.

Fidalgo, B.V., and V.A. Najjar. 1967. The physiological role of the lymphoid system. III. Leucophilic γ-globulin and the phagocytic activity of the polymorphonuclear leucocytes. Proc. Natl. Acad. Sci. 57:957.

Fiser, A., and K. Svitavsky. 1973. Zum Vorkommen der hämolytischen Stämme Escherichia coli und Staphylococcus im Luftmilieu der Grosszuchtanlagen für Schweine und Milchkühe im Verlauf eines Jahres. Wiss. Z. Karl-Marx-Univ. Lpz. 22:508.

Forbes, D., and C.N. Herbert. 1968. Studies in the pathogenesis of staphylococcal mastitis. Vet. Rec. 82:69.

Fox, L.K., J.S. McDonald, J.K. Hillers, and L.B. Corbeil. 1988. Function of phagocytes obtained from lacteal secretions of lactating and nonlactating cows. Am. J. Vet. Res. 49: 678.

Frost, A.J. 1975. Selective adhesion of microorganisms to the ductular epithelium of the bovine mammary gland. Infect. Immun. 12:1154.

Frost, A.J., D.D. Wanasinghe, and J.B. Woolcock. 1977. Some factors affecting selective adherence of microorganisms in the bovine mammary gland. Infect. Immun. 15:245.

Fullerton, F.M., I.R. Fleet, R.B. Heap, I.C. Hart, and T.B. Mepham. 1989. Cardiovascular responses and mammary substrate uptake in Jersey cows treated with pituitary-derived growth hormone during late lactation. J. Dairy Res. 56:27.

Girard, F. 1984. Hormone de croissance - données physiologiques. Bull. Acad. Nat. Méd. 168:534.

Gommard, E., J.P. Levy, F. Plata, Y. Henn, V. Duprez, A. Bismuth, T. Reme. 1978. Studies on the nature of the cell surface antigen reacting with cytotoxic T lymphocytes in murine oncogenic virus induced tumours. Eur. J. Immunol. 8:228.

Gray, G.D., K.A. Knight, R.D. Nelson, and M.J. Herron. 1982. Chemotactic requirements of bovine leukocytes. Am. J. Vet. Res. 43:757.

Greer, D.O., and J.K.L. Pearson. 1976. Reproducibility of electronic cell counts in milk; a study of 5 further factors. J. Dairy Res. 43:371.

Guidry, A.J., M. Ost, I.H. Mather, W.E. Shainline, and B.T. Weinland. 1983. Sequential response of milk leukocytes, albumin, immunoglobulins, monovalent ions, citrate, and lactose in cows given infusions of Escherichia coli endotoxin into the mammary gland. Am. J. Vet. Res. 44:2262.

Guidry, A.J., and R.H. Miller. 1986. Immunoglobulin isotype concentrations in milk as affected by stage of lactation and parity. J. Dairy Sci. 69:1799.

Guillemin, R., P. Brazeau, P. Böhlen, F. Esch, N. Ling, and W.B. Wehrenberg. 1982. Growth hormone - releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585.

Haire, R.N., M.S. Peterson, J.J. O'Leary. 1988. Mitogen activation induces the enhanced synthesis of two heat-shock proteins in human lymphocytes. J. Cell Biol. 106:883.

Harmon, R.J., and C.W. Heald. 1982. Migration of polymorphonuclear leukocytes into the bovine mammary gland during experimentally induced Staphylococcus aureus mastitis. Am. J. Vet. Res. 43:992.

Harp, J.A., and B.J. Nonnecke. 1986. Regulation of mitogenic responses by bovine milk leukocytes. Vet. Immunol. Immunopathol. 11:125.

Hart, I.C., J.A. Bines, S. James, and S.V. Morant. 1985. The effect of injecting or infusing low doses of bovine growth hormone on milk yield, milk composition and the quantity of hormone in the milk serum of cows. Anim. Prod. 40:243.

Hart, I.C., J.A. Bines, and S.V. Morant. 1980. The secretion and metabolic clearance rates of growth hormone, insulin and prolactin in high- and low-yielding cattle at four stages of lactation. Life Sci. 27:1839.

Hayashi, H., M. Yoshinaga, and S. Yamamoto. 1974. The nature of a mediator of leukocyte chemotaxis in inflammation. Antibiot. Chemother. 19:296.

Hayashida, T., and C.H. Li. 1957. The influence of adrenocorticotropic and growth hormones on antibody formation. J. Exp. Med. 105:93.

Heald, C.W., G.M. Jones, S.C. Nickerson, W.N. Patterson, and W.E. Vinson. 1977. Preliminary evaluation of the Fossomatic cell counter for analysis of individual cow samples in a central testing laboratory. J. Food Prot. 40:523.

Heeschen, W. 1975. Determination of somatic cells in milk (technical aspects of counting). Page 79 in Proc. Seminar on Mastitis Control, International Dairy Federation. Bull. Doc. 85.

Heyneman, R., C. Burvenich, M. Van Hoegaerden, and G. Peeters. 1989. Influence of recombinant methionyl bovine somatotropin (rBST) on blood neutrophil respiratory burst activity in healthy cows. J. Dairy Sci. 72(Suppl.):349.(Abstr.)

Hill, A.W., A.L. Shears, and K.G. Hibbitt. 1978. The elimination of serum-resistant Escherichia coli from experimentally infected single mammary glands of healthy cows. Res. Vet. Sci. 25:89.

Hoare, R.J.T., P.J. Nicholls, and R.F. Sheldrake. 1982. Investigations into falsely elevated somatic cell counts of bulked herd milk. J. Dairy Res. 49:559.

Hogan, J.S., K.L Smith, W.P. Weiss, D.A. Todhunter, and P.S. Schoenberger. 1989. Relationships among polymorphonuclear leukocyte functions and dietary vitamin E and selenium supplementation in dairy cows. J. Dairy Sci. 71(Suppl.1):10.(Abstr.)

Holmberg, O., and C. Concha. 1985. The function of leucocytes in mammary secretion. Kieler Milchwirtschaftl. Forschungsber. 37:458.

Honkanen-Buzalski, T., R. Kangasniemi, F. Astroshi, and M. Sandholm. 1981. Effect of lactation stage and number on milk albumin (BSA) and somatic cell count. Zbl. Vet. Med. A. 28:760.

Honkanen-Buzalski, T. and M. Sandholm. 1981. Association of bovine secretory immunoglobulins with milk fat globule membranes. Comp. Immun. Microbiol. Infect. Dis. 4:329.

Hurley, D.J., M.H. Kensinger, A.M. Mastro, and R.A. Wilson. 1990. An evaluation of the mononuclear cells derived from bovine mammary gland dry secretions using leukocyte specific monoclonal antibodies, light scattering properties, and non-specific esterase staining. Vet. Immunol. Immunopathol. (submitted).

Igono, M.O., H.D. Johnson, B.J. Steevens, W.A. Hainen, and M.D. Shanklin. 1988. Effect of season on milk temperature, milk growth hormone, prolactin, and somatic cell counts of lactating cattle. Int. J. Biometeorol. 32:194.

International Dairy Federation. 1981. Laboratory methods for use in mastitis work. Document 132. Brussels, Belgium.

Jain, N.C., E.J. Carroll, and O.W. Schalm. 1968. Influence of antiserum to bovine leukocytes on circulating leukocytes of the cow. Am. J. Vet. Res. 29:2081.

Jampel, H.D., G.W. Duff, R.K. Gershon, E. Atkins, and S.K. Durum. 1983. Fever and immunoregulation. III. Hyperthermia augments the primary in vitro humoral immune response. J. Exp. Med. 157:1229.

Johnson, H.D., B.A. Becker, K.J. Spencer, R.J. Collier, and C.A. Baile. 1988. Effects of field and laboratory heat stress on milk and physiological parameters of lactating dairy cows supplemented with sometribove (methionyl bovine somatotropin, BST). J. Dairy Sci.(Suppl.1):124.(Abstr.)

Kaczmarek, L., B. Calabretta, H.T. Kao, N. Heintz, J. Nevins, and R. Baserga. 1987. Control of hsp70 RNA levels in human lymphocytes. J. Cell Biol. 104:183.

Kalland, T., and I. Dahlquist. 1983. Effects of in vitro hyperthermia on human natural killer cells. Cancer Res. 43:1842.

Kehrli, M.E., Jr., J.A. Harp, J.P. Goff, J.R. Stabel, J.R. Thurston, M.G. Stevens, D.J. Hurley, R.A. Wilson, and T.C. Boone. 1990. Effects of recombinant bovine G-CSF administration on bacterial shedding and leukocyte functions in periparturient dairy cows. Am. J. Vet. Res. (submitted).

Keller, H.U., P.C. Wilkinson, M. Abercrombie, E.L. Becker, J.G. Hirsch, M.E. Miller, W.S. Ramsey, and S.H. Zigmond. 1977. A proposal for the definition of terms related to locomotion of leukocytes and other cells. Clin. Exp. Immunol. 27:377.

Kelley, K.W., R.E. Greenfield, J.F. Evermann, S.M. Parish, and L.E. Perryman. 1982a. Delayed-type hypersensitivity, contact sensitivity, and phytohemagglutinin skin-test responses of heat- and cold-stressed calves. Am. J. Vet. Res. 43:775.

Kelley, K.W., C.A. Osborne, J.F. Evermann, S.M. Parish, and C.T. Gaskins. 1982b. Effects of chronic heat and cold stressors on plasma immunoglobulin and mitogen-induced blastogenesis in calves. J. Dairy Sci. 65:1514.

Kennedy, B.W., M.S. Sethar, A.K.W. Tong, J.E. Moxley, and B.R. Downey. 1982. Environmental factors influencing test-day somatic cell counts in Holsteins. J. Dairy Sci. 65:275.

Kiess, W., and O. Butenandt. 1985. Specific growth hormone receptors on human peripheral mononuclear cells: Reexpression, identification, and characterization. J. Clin. Endocrinol. Metab. 60:740.

Kiess, W., and O. Butenandt. 1987. Regulation of GH binding to specific cellular receptors in vitro--A new model of growth regulation in vivo. Horm. Metab. Res. 19:171.

Kiess, W., S. Malozowski, M. Gelato, O. Butenandt, H. Doerr, B. Crisp, E. Eisl, A. Maluish, and B.H. Belohradsky. 1988. Lymphocyte subset distribution and natural killer activity in growth hormone deficiency before and during short-term treatment with growth hormone releasing hormone. Clin. Immunol. Immunopathol. 48:85.

Kitchen, B.J., and G. Middleton. 1975. A rapid enzymatic test for the estimation of the somatic cell count in bovine milk. Australian Vet. J. 52:102.

Lanzavecchia, A. 1987. Antigen uptake and accumulation in antigen-specific B cells. Immunol. Rev. 99:37.

Lapierre, H., G. Pelletier, D. Petitclerc, P. Dubreuil, J. Morisset, P. Gaudreau, Y. Couture, and P. Brazeau. 1988. Effect of two-month treatment with growth hormone releasing factor on growth hormone release in dairy cows. Can. J. Anim. Sci. 68:731.

Lee, C.-S., F.B.P. Wooding and P. Kemp. 1980. Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows. J. Dairy Res. 47:39.

Lentnek, A.L., A.D. Schreiber, and R.R. MacGregor. 1976. The induction of augmented granulocyte adherence by inflammation. J. Clin. Invest. 57:1098.

Lesniak, M.A., P. Gorden, J. Roth, and J.R. Gavin, III. 1974. Binding of ^{125}I-human growth hormone to specific receptors in human cultured lymphocytes. J. Biol. Chem. 249:1661.

Lidwell, O.M., and E.J. Lowbury. 1950. The survival of bacteria in dust. II. The effect of atmospheric humidity on the survival of bacteria in dust. J. Hyg.(Camb.) 48:21.

Lintner, T.J., and R.J. Eberhart. 1987. Effects of antibiotics and dry period mammary secretion on bovine polymorphonuclear neutrophil (PMN) function and morphology. J. Dairy Sci. 70(Suppl1):212.(Abstr.)

Linzell, J.L., M. Peaker, and J.G. Rowell. 1974. Electrical conductivity of foremilk for detecting subclinical mastitis in cows. J. Agric. Sci.(Camb.) 83:309.

Loertscher, R., M. Abbud-Filho, A.B. Leichtman, A.A. Ythier, J.M. Williams, C.B. Carpenter, and T.B. Strom. 1987. Differential effect of gamma-irradiated and heat-treated lymphocytes on T cell activation, and interleukin-2 and interleukin-3 release in the human mixed lymphocyte reaction. Transplantation 44:673.

Lough, D.S., L.D. Muller, R.S. Kensinger, L.C. Griel, Jr., and C.D. Azzara. 1989. Effect of exogenous bovine somatotropin on mammary lipid metabolism and milk yield in lactating dairy cows. J. Dairy Sci. 72:1469.

Mallonée, P.G., D.K. Beede, R.J. Collier, and C.J. Wilcox. 1985. Production and physiological responses of dairy cows to varying dietary potassium during heat stress. J. Dairy Sci. 68:1479.

Manalu, W., H.D. Johnson, B.A. Becker, R. Li, E.B. Abdalla, P.S. Katti, and R.J. Collier. 1988. Effects of sometribove (methionyl bovine somatotropin, BST) on heat balance during heat and cold exposure in lactating cattle. J. Dairy Sci.(Suppl.1):124.(Abstr.)

Marple, D.N., E.D. Aberle, J.C. Forrest, W.H. Blake, and M.D. Judge. 1972. Effects of humidity and temperature on porcine plasma adrenal corticoids, ACTH and growth hormone levels. J. Anim. Sci. 34:809.

Mattila, T. 1985. Diagnostic Problems in bovine mastitis. Academic Dissertation, College of Veterinary Medicine, Helsinki, Finland.

McDade, J.J., L.B. Hall, and A.K. Street. 1963. An experimental method to measure the influence of environmental factors on the viability and the pathogenicity of Staphylococcus aureus. Am. J. Hyg. 77:98.

McDermott, M.P., H.N. Erb, and R.P. Natzke. 1982. Predictability by somatic cell counts related to prevalence of intramammary infection within herds. J. Dairy Sci. 65:1535.

McDonald, J.S. 1971a. Bovine udder infection: Time of microbial invasion of the teat sinus. Page 91 in Proc. VI Int. Conf. Cattle Dis., Stillwater, OK.

McDonald, J.S. 1971b. Microscopic observations of teat canals from susceptible and resistant bovine mammary glands: A preliminary report. Page 97 in Proc. VI Int. Conf. Cattle Dis., Stillwater, OK.

McDowell, R.E. 1972. Improvement of livestock production in warm climates. Freeman, San Francisco Press, CA.

McGillen, J., R. Patterson, and J.P. Phair. 1980. Adherence of polymorphonuclear leukocytes to nylon: Modulation by prostacyclin (PGI2), corticosteroids, and complement activation. J. Infect. Dis. 141:382.

McGuffey, R.K., H.B. Green, R.P. Basson, and T.H. Ferguson. 1990. Lactation response of dairy cows receiving bovine somatotropin via daily injections or in a sustained-release vehicle. J. Dairy Sci. 73:763.

McGuire, T.C., A.J. Musoke, and T. Kurtti. 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology 38:249.

McMichael, A. 1978. HLA restriction of human cytotoxic T lymphocytes specific for influenza virus. Poor recognition of virus associated with HLA A2. J. Exp. Med. 148:1458.

Merriam, G.R., and K.W. Wachter. 1982. Algorithms for the study of episodic hormone secretion. Am. J. Physiol. 243:E310.

Miller, R.H., M.J. Paape, and J.C. Acton. 1986. Comparison of milk somatic cell counts by Coulter and Fossomatic counters. J. Dairy Sci. 69:1942.

Miller, R.H., M.J. Paape, A.M. Dulin, W.D. Schultze, B.T. Weinland, and R.E. Pearson. 1985. Factors affecting abililty of skim milk to support phagocytosis by bovine polymorphonuclear leukocytes. J. Dairy Sci. 68:3087.

Mitra, R., G.I. Christison, and H.D. Johnson. 1972. Effect of prolonged thermal exposure on growth hormone (GH) secretion in cattle. J. Anim. Sci. 34:776.

Mizzen, L.A., and W.J. Welch. 1988. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. J. Cell Biol. 106:1105.

Mohammed, M.E., and H.D. Johnson. 1985. Effect of growth hormone on milk yields and related physiological functions of Holstein cows exposed to heat stress. J. Dairy Sci. 68:1123.

Morse, D., M.A. DeLorenzo, R.P. Natzke, and D.R. Bray. 1988. Characterization of clinical mastitis records from one herd in a subtropical environment. J. Dairy Sci. 71:1396.

Morse, G.E., K. Hubben, and K. Mitchell. 1971. The teat canal as a natural barrier to udder infection. Page 108 in Proc. VI Int. Conf. Cattle Dis., Stillwater, OK.

Moseley, W.M., L.F. Krabill, A.R. Friedman, and R.F. Olsen. 1984. Growth hormone response of steers injected with synthetic human pancreatic growth hormone-releasing factors. J. Anim. Sci. 58:430.

Mueller, R., E.J. Carroll, and L. Panico. 1983. Hemolytic complement titers and complement C3 levels in endotoxin-induced mastitis. Am. J. Vet. Res. 44:1442.

Muscoplat, C.C., R.E. Shope, Jr., A.W. Chen, and D.W. Johnson. 1975. Effects of corticosteroids on responses of bovine peripheral blood lymphocytes cultured with phytohemagglutinin. Am. J. Vet. Res. 36:1243.

Nagy, E., and I. Berczi. 1978. Immunodeficiency in hypophysectomized rats. Acta Endocrinol. 89:530.

Nagy, E., I. Berczi, and H.G. Friesen. 1983. Regulation of immunity in rats by lactogenic and growth hormones. Acta Endocrinol. 102:351.

Närvänen, O., I. Jokinen, K. Poikonen, L. Rasanen, and H. Arvilommi. 1986. Effect of elevated temperature on human immunoglobulin synthesis, lymphokine production and lymphocyte proliferation. Acta Path. Microbiol. Immunol. Scand. C94:239.

Natzke, R.P. 1981. Elements of mastitis control. J. Dairy Sci. 64:1431.

Natzke, R.P., and R.W. Everett. 1975. The elimination of mastitis by culling. Page 303 in Proc. Seminar on Mastitis Control, International Dairy Federation. Bull. Doc. 85.

Natzke, R.P., R.W. Everett, R.S. Guthrie, J.F. Keowin, A.M. Meek, W.G. Merrill, S.J. Roberts, and G.H. Schmidt. 1972. Mastitis Control Program: Effect on milk production. J. Dairy Sci. 55:1256.

Natzke, R.P., R.W. Everett, and D.S. Postle. 1972. Normal milk somatic cell counts. J. Milk Food Technol. 35:261.

Nelson, R.D., P.G. Quie, and R.L. Simmons. 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115:1650.

Neville, A.J., and D.N. Sauder. 1988. Whole body hyperthermia (41-42°C) induces interleukin-1 in vivo. Lymphokine Res. 7:201.

Newbould, F.H.S. 1973. The effect of added serum and glucose and some inherent factors on phagocytosis in vitro by milk leukocytes from several cows. Can. J. Comp. Med. 37:189.

Newby, T.J., and J. Bourne. 1977. The nature of the local immune system of the bovine mammary gland. J. Immunol. 118:461.

Nickerson, S.C., P.A. Baker, and P. Trinidad. 1989. Local immunostimulation of the bovine mammary gland with interleukin-2. J. Dairy Sci. 72:1764.

Nickerson, S.C., W.E. Owens, and J.L. Watts. 1989. Effects of recombinant granulocyte colony-stimulating factor on staphylococcus aureus mastitis in lactating dairy cows. J. Dairy Sci. 72:3286.

Nickerson, S.C., M.J. Paape, and A.M. Dulin. 1985. Effect of antibiotics and vehicles on bovine mammary polymorphonuclear leukocyte morphologic features, viability, and phagocytic activity in vitro. Am. J. Vet. Res. 46:2259.

Nickerson, S.C., M.J. Paape, R.J. Harmon, and G. Ziv. 1986. Mammary leukocyte response to drug therapy. J. Dairy Sci. 69:1733.

Nickerson, S.C., and J.W. Pankey. 1984. Neutrophil migration through teat end tissues of bovine mammary quarters experimentally challenged with Staphylococcus aureus. J. Dairy Sci. 67:826.

Nickerson, S.C., and J.W. Pankey. 1983. Cytologic observations of the bovine teat end. Am. J. Vet. Res. 44:1433.

Nonnecke, B.J., and J.A. Harp. 1985. Effect of chronic staphylococcal mastitis on mitogenic responses of bovine lymphocytes. J. Dairy Sci. 68:3323.

Nonnecke, B.J., and J.A. Harp. 1989. Function and regulation of lymphocyte-mediated immune responses: Relevance to bovine mastitis. J. Dairy Sci. 72:131.

Norcross, N.L., and J.P. Opdebeeck. 1983. Encapsulation of Staphylococcus aureus isolated from bovine milk. Vet. Microbiol. 8:397.

Ojo-Amaize, E.A., A.J. Guidry, M. Paape, and H.K. Mayer. 1988. In vitro depression of bovine lymphocyte function by treatment of cultured bovine lymphocytes with physiologic concentrations of hydrocortisone. Am. J. Vet. Res. 49:851.

Oldham, E.R., M.J. Daley, D.A. D'Angelo, S.R. Riddle and T.J. Williams. 1989. Quantitative and qualitative properties of bovine polymorphonuclear neutrophils during Staphylococcus aureus infection. J. Dairy Sci. 71(Suppl.1):10.(Abstr.)

Oliver, S.P., and B.A. Mitchell. 1983. Susceptibility of bovine mammary gland to infections during the dry period. J. Dairy Sci. 66:1162.

Omar, R.A., S. Yano, and Y. Kikkawa. 1987. Antioxidant enzymes and survival of normal and Simian virus 40-transformed mouse embryo cells after hyperthermia. Cancer Res. 47:3473.

Opdebeeck, J.P., and N.L. Norcross. 1982. Antibody response in lacteal secretions of cows after immunization with various concentrations of staphylococcal and streptococcal antigens. Am. J. Vet. Res. 43:1770.

Paape, M.J., A.J. Guidry, K.E. Squiggins, and R.H. Miller. 1989. Identification of four bovine neutrophil subpopulations using antineutrophil monoclonal antibodies. Proc. Fed. Am. Soc. Exp. Biol. 47:(Abstract).

Paape, M.J., R.E. Pearson, W.P. Wergin, and A.J. Guidry. 1977. Enhancement of chemotactic response of polymorphonuclear leukocytes into the mammay gland and isolation from milk. J. Dairy Sci. 60:53.

Paape, M.J., W.D. Schultze, R.H. Miller, and J.W. Smith. 1973. Thermal stress and circulating erythrocytes, leukocytes, and milk somatic cells. J. Dairy Sci. 56:84.

Paape, M.J., W.P. Wirgin, A.J. Guidry and R.E. Pearson. 1979. Leukocytes--second line of defense against invading mastitis pathogens. J. Dairy Sci. 62:135.

Pabst, M.J., and R.B. Johnston, Jr. 1980. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. J. Exp. Med. 151:101.

Palmiter, R.D., G. Norstedt, R.E. Gelinas, R.E. Hammer, and R.L. Brinster. 1983. Metallothionein-Human GH fusion genes stimulate growth of mice. Science 222:809.

Pankey, J.W., N.T. Boddie, J.L. Watts, and S.C. Nickerson. 1985. Evaluation of protein A and a commercial bacterin as vaccines against Staphylococcus aureus mastitis by experimental challenge. J. Dairy Sci. 68:726.

Parkhie, M.R., and H.D. Johnson. 1969. Growth hormone releasing activity in the hypothalamus of rats subjected to prolonged heat stress. Proc. Soc. Exp. Biol. Med. 130:843.

Peel, C.J., D.E. Bauman, R.C. Gorewit, and C.J. Sniffen. 1981. Effect of exogenous growth hormone on lactational performance in high yielding dairy cows. J. Nutr. 111:1662.

Phillips, T.R., W.C. Yang, and R.D. Schultz. 1987. The effects of glucocorticosteriods on the chemiluminescence response of bovine phagocytic cells. Vet. Immunol. Immunopathol. 14:245.

Postle, D.S., M. Roguinsky, and B. Poutrel. 1978. Induced staphylococcal infections in the bovine mammary gland. Am. J. Vet. Res. 39:29.

Prescott, S.C., and R.S. Breed. 1910. The determination of the number of body cells in milk by a direct method. J. Infect. Dis. 7:632.

Quaife, C.J., L.S. Mathews, C.A. Pinkert, R.E. Hammer, R.L. Brinster, and R.D.Palmiter. 1989. Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice. Endocrinology 124:40.

Rainard, P. 1985. Assessment of attachment, ingestion, and killing of Escherichia coli by bovine polymorphonuclear cells with combined micromethods. Vet. Immunol. Immunopathol. 10:155.

Rainard, P., and B. Poutrel. 1982. Dynamics of nonclinical bovine intramammary infections with major and minor pathogens. Am. J. Vet. Res. 43:2143.

Rajkovic, I.A., and R. Williams. 1985. Rapid microassays of phagocytosis, bacterial killing, superoxide and hydrogen peroxide production by human neutrophils in vitro. J. Immunol. Methods 78:35.

Rapaport, R., J. Oleske, H. Ahdieh, K. Skuza, B.K. Holland, M.R. Passannante, and T.Denny. 1987. Effects of human growth hormone on immune functions: In vitro studies on cells of normal and growth hormone-deficient children. Life Sci. 41:2319.

Rapaport, R., J. Oleske, H. Ahdieh, S. Solomon, C. Delfaus, and T. Denny. 1986. Suppression of immune function in growth hormone-deficient children during treatment with human growth hormone. J. Pediatr. 109:434.

Reddy, P.G., F. Blecha, D.S. McVey, J.E. Shirley, J.L. Morrill and P.E. Baker. 1989. Bovine recombinant granulocyte-macrophage colony-stimulating factor augments functions of neutrophils from mastitic cows. J. Dairy Sci. 71(Suppl.):11.(Abstr.)

Regnier, J.A., and K.W. Kelley. 1981. Heat-and cold-stress supresses in vivo and in vitro cellular immune responses of chickens. Am. J. Vet. Res. 42:294.

Reinherz, E.L. and S.F. Schlossman. 1980. The differentiation and functions of human T-cells. Cell. 19:821.

Riabowol, K.T., L.A. Mizzen, and W.J. Welch. 1988. Heat shock is lethal to fibroblasts microinjected with antibodies against hsp 70. Science 242:433.

Richard, A.L., S.N. McCutcheon, and D.E. Bauman. 1985. Responses of dairy cows to exogenous bovine growth hormone administered during early lactation. J. Dairy Sci. 68:2385.

Roberts, N.J., Jr. 1986. Differential effects of hyperthermia on human leukocyte production interferon-α and interferon-γ (42383). Proc. Soc. Exp. Biol. Med. 183:42.

Roberts, N.J., Jr., and K. Sandberg. 1979. Hyperthermia and human leukocyte function. II. Enhanced production of and response to leukocyte migration inhibition factor (LIF). J. Immunol. 122:1990.

Roberts, N.J., Jr., and R.T. Steigbigel. 1977. Hyperthermia and human leukocyte functions: Effects on response of lymphocytes to mitogen and antigen and bactericidal capacity of monocytes and neutrophils. Inf. Imm. 18:673.

Rodbard, S. 1950. Weight and body temperature. Science 111:465.

Rodriquez, L.A., G. Medonnen, C.J. Wilcox, F.G. Martin and W.A. Krienke. 1985. Effects of relative humidity, maximum and minimum temperature, pregnancy, and stage of lactation on milk composition and yield. J. Dairy Sci. 68:973.

Roitt, I. 1980. Essential Immunology. Blackwell Scientific Publications, Oxford, United Kingdom.

Roman-Ponce, H., W.W. Thatcher, D.E. Buffington, C.J. Wilcox, and H.H. Van Horn. 1977. Physiological and production responses of dairy cattle to a shade structure in a subtropical environment. J. Dairy Sci. 60:424.

Rose, D., W.J. Welch, G. Kramer, and B. Hardesty. 1989. Possible involvement of the 90-kDa heat shock protein in the regulation of protein synthesis. J. Biol. Chem. 264:6239.

Roth, J.A., and M.L. Kaeberle. 1981. Effects of in vivo dexamethasone administration on in vitro bovine polymorphonuclear leukocyte function. Infect. Immun. 33:434.

Roth, J.A., M.L. Kaeberle, and W.H. Hsu. 1982. Effects of ACTH administration on bovine polymorphonuclear leukocyte function and lymphtocyte blastogenesis. Am J. Vet. Res. 43:412.

Rothman, K.J. 1986. Modern Epidemiology. Little, Brown, and Company, Boston, MA.

Rovensky, J., M. Vigas, J. Lokaj, P. Cuncik, P. Lukac, and A. Takac. 1982. Effect of growth hormone on the metabolic activity of phagocytes of peripheral blood in pituitay dwarfs and acromegaly. Encdocrinol. Exp. 16:129.

Rullmann, W., and R. Trommsdorff. 1906. Milchhygienische Untersuchungen. Arch. f. Hyg. 59:224.

SAS Institute, Inc. 1982. SAS User's Guide: Statistics. Ed. SAS Inst., Inc., Cary, NC.

Savage, W.G. 1906. Streptococci and leucocytes in milk. I. J. Hyg. 6:123.

Schalm, O.W., J. Lasmanis, and N.C. Jain. 1976. Conversion of chronic staphylococcal mastitis to acute gangrenous mastitis after neutropenia in blood and bone marrow produced by an equine anti-bovine leukocyte serum. Am. J. Vet. Res. 37:885.

Schams, D., E. Stephan, and R.D. Hooley. 1980. The effect of heat exposure on blood serum levels of anterior pituitary hormones in calves, heifers and bulls. Acta Endocrinol. 94:309.

Schlesinger, M.J. 1988. Function of heat shock proteins. ISI Atlas Science: Biochemistry 1:161.

Schmidt, J.A., and E. Abdulla. 1988. Down-regulation of IL-1 biosynthesis by inducers of the heat-shock response. J. Immunol. 141:2027.

Schmidt-Madsen, P.S. 1975. Fluoro-opto-electronic cell counting of milk. Page 133 in Proc. Seminar on Mastitis Control, International Dairy Federation. Bull. Doc. 85.

Schneider, P.L., D.K. Beede, C.J. Wilcox, and R.J. Collier. 1984. Influence of dietary sodium and potassium bicarbonate and total potassium on heat-stressed lactating cows. J. Dairy Sci. 67:2546.

Schore, C.E., B.I. Osburn, D.E. Jasper, and D.E. Tyler. 1981. B and T lymphocytes in the bovine mammary gland: rosette formation and mitogen response. Vet. Immunol. Immunopathol. 2:561.

Sheldrake, R.F., R.J.T. Hoare, and G.D. McGregor. 1983. Lactation state, parity, and infection affecting somatic cells, electrical conductivity, and serum albumin in milk. J.Dairy Sci. 66:542.

Sheldrake, R.F., G.D. McGregor, and R.J.T. Hoare. 1983. Somatic cell count, electrical conductivity, and serum albumin concentration for detecting bovine mastitis. J. Dairy Sci. 66:548.

Smal J., J. Closset, G. Hennen, and P. de Meyts. 1987. Receptor binding properties and insulin-like effects of human growth hormone and its 20 kDa-variant in rat adipocytes. J. Biol. Chem. 262:11071.

Smith, J.W., and R.D. Schultz. 1977. Mitogen- and antigen-responsive milk lymphocytes. Cell. Immunol. 29:165.

Smith, K.A., and F.W. Rusceti. 1981. T-cell growth factor and the culture of cloned functional T-cells. Adv. Immunol. 31:137.

Soderholm, C.G., D.E. Otterby, J.G. Linn, F.R. Ehle, J.E. Wheaton, W.P. Hansen, and R.J. Annexstad. 1988. Effects of recombinant bovine somatotropin on milk production, body composition, and physiological parameters. J. Dairy Sci. 71:355.

Steward, M. 1989. Antigen recognition. Page 7.1 in Immunology. 2nd ed. I.M. Roitt, J.Brostoff and D.K. Male, ed. Gower Medical Publishing, London, New York.

Stott, G.H., F. Wiersma, B.E. Menefee, and F.R. Radwanski. 1976. Influence of environment on passive immunity in calves. J. Dairy Sci. 59:1307.

Sweetsur, A.W.M., and J.D. Phillips. 1976. Effect of storage on the electronic cell count of milk. J. Dairy Res. 43:53.

Szijarto, L.F., and D.A. Barnum. 1984. Preparation, use and evaluation of standards developed for simultaneous monitoring of Coulter and Fossomatic electronic cell counting instruments in Ontario. J. Food Prot. 47:227.

Targowski, S.P., and M. Niemialtowski. 1986. Inhibition of lacteal leukocyte phagocytosis by colostrum, nonlactating secretion, and mastitic milk. Am. J. Vet. Res. 47:1940.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka, and L.R. Daniel. 1988a. Bovine vitamin A and β-carotene intake and lactational status. 1. Responsiveness of peripheral blood polymorphonuclear leukocytes to vitamin A and β-carotene challenge in vitro. J. Dairy Sci. 71:3112.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka, and L.R. Daniel. 1988b. Bovine vitamin A and β-carotene intake and lactational status. 2. Responsiveness of mitogen-stimulated peripheral blood lymphocytes to vitamin A and β-carotene challenge in vitro. J. Dairy Sci. 71:3120.

Trenkle, A. 1972. Growth hormone secretion rates in cattle. J. Anim. Sci. 32:115.

Vicini, J.L., J.M. De Leon, W.J. Cole, P.J. Eppard, G.M. Lanza, S. Hudson, and M.A. Miller. 1988. Effect of acute administration of extremely large doses of sometribove, USAN (recombinant methionyl bovine somatotropin), in a prolonged release formulation on milk production and health of dairy cows. J. Dairy Sci. 71(Suppl.1):168.(Abstr.)

Ward, G.E., and L.H. Schultz. 1972. Relationship of somatic cells in quarter milk to type of bacteria and production. J. Dairy Sci. 55:1428.

Washburn, M., P.H. Klesius, and K.G. Venkataseshu. 1982. Characterization of the chemiluminescence response of equine phagocytes. Am. J. Vet. Res. 43:1147.

Watson, D.L. 1975. Cytophilic attachment of ovine IgG2 to autologous polymorphonuclear leucocytes. Aust. J. Exp. Biol. Med. Sci. 53:527.

Watson, D.L. 1976. The effect of cytophilic IgG2 on phagocytosis by ovine polymorphonuclear leucocytes. Immunology 31:159.

Watson, D.L. 1984. Evaluation of attenuated, live staphylococcal mastitis vaccine in lactating heifers. J. Dairy Sci. 67:2608.

Watson, D.L., and H.I. Davies. 1985. Immunophysiological activity of supramammary lymph nodes of the ewe during the very early phase of staphylococcal mastitis. Res. Vet. Sci. 39:52.

Weber, L., E. Peterhans, and R. Wyler. 1983. The chemiluminescent response of bovine polymorphonuclear leucocytes isolated from milk and blood. Vet. Immunol. Immunopathol. 4:397.

Webster, A.J.F. 1981. Weather and infectious disease in cattle. Vet. Rec. 108:183.

Wegner, T.N., J.D. Schuh, F.E. Nelson, and G.H. Stott. 1976. Effect of stress on blood leucocyte and milk somatic cell counts in dairy cows. J. Dairy Sci. 59:949.

Wegner, T.N., and G.H. Stott. 1971. Effects of stress on abnormal milk production. J. Dairy Sci. 54(Suppl.1):798. (Abstr.)

Weigent, D.A., J.B. Baxter, W.E. Wear, L.R. Smith, K.L. Bost, and J.E. Blalock. 1988. Production of immunoreactive growth hormone by mononuclear leukocytes. FASEB J. 2:2812.

Weiss, W.P., J.S. Hogan, K.L. Smith and K.H. Hoblet. 1990. Relationships among selenium, vitamin E, and mammary gland health in commercial dairy herds. J. Dairy Sci. 73:381.

Welch, W.J., L.A. Mizzen, and A.-P. Arrigo. 1989. Structure and function of mammalian stress proteins. Page 187 in Stress-Induced Proteins. M.L. Pardue, J.R. Feramisco, and S. Lindquist, ed. A.R. Liss, New York, NY.

West, J.W., K. Bondari, and J.C. Johnson, Jr. 1989. The response of lactating Holstein and Jersey cows to recombinant bovine somatotropin (rbST) administered during hot, humid weather. J. Dairy Sci. 72(Suppl.1):427.(Abstr.)

Whitfield, J.F., J.P. MacManus and R.H. Rixon. 1971. Stimulation by growth hormone of deoxyribonucleic acid synthesis and proliferation of rat thymic lymphocytes. Horm. Metab. Res. 3:28.

Wilson, R.A., J.A. Linn, and R.J. Eberhart. 1986. A study of bovine T-cell subsets in the blood and mammary gland secretions during the dry period. Vet. Immunol. Immunopathol. 13:151.

Wise, M.E., D.V. Armstrong, J.T. Huber, R. Hunter, and F. Wiersma. 1988. Hormonal alterations in the lactating dairy cow in response to thermal stress. J. Dairy Sci. 71:2480.

Woolford, M.W. 1985. The relationship between mastitis and milk yield. Kieler Milchwirtschaftl. Forschungsber. 37:224.

Wray, C. 1975. Survival and spread of pathogenic bacteria of veterinary importance within the environment. Vet. Bull. 45:543.

Yang, T.J., J.F. Mather and E.D. Rabinovsky. 1988. Changes in subpopulations of lymphocytes in peripheral blood, and supramammary and prescapular lymph nodes of cows with mastitis and normal cows. Vet. Immunol. Immunopath. 18:279.

Yousef, M.K., and H.D. Johnson. 1966. Calorigenesis of cattle as influenced by growth hormone and environmental temperature. J. Anim. Sci. 25:1076.

Yousef, M.K., Y. Takahashi, W.D. Robertson, L.J. Machlin and H.D. Johnson. 1969. Estimation of growth hormone secretion rate in cattle. J. Anim. Sci. 29:341.

Zinkernagel, R.M. and P.C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature 248:701.

Zoe-Mboe, A., H.H. Head, K.C. Bachman, F. Baccari, Jr., and C.J. Wilcox. 1989. Effects of bovine somatotropin on milk yield and composition, dry matter intake, and some physiological functions of Holstein cows during heat stress. J. Dairy Sci. 72:907.

BIOGRAPHICAL SKETCH

Francois Elvinger was born September 12, 1956, in Luxembourg, Luxembourg, as the second son to Francois Elvinger and Monique Goerens. He received his primary and secondary education in Luxembourg and in 1975 graduated from the Latin-Mathematics section of the Athénée Grande-Duchesse Charlotte in Luxembourg. From 1975 to 1983 he attended the Veterinary School in Hannover, West-Germany, to graduate as veterinary surgeon in 1981, and to acquire the degree of Dr. med. vet. in 1983, after completion of his dissertation on prevalence of Streptococcus agalactiae and hygiene factors in dairy herds. Before joining the Dairy Science Department in January, 1986, he worked as scientific assistant at the Milk Hygiene and Technology Institute of the Hannover Veterinary School. In 1986, he married his wife Dawn Dykehouse, who brought her daughter Maghan into their marriage, and the family has recently been expanded by a new addition, Kristin, born March 29, 1990.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. Roger P. Natzke, Chair
Professor of Animal Science

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Dr. R. Kenneth Braun
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August, 1990

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